

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/10574

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/57 C12N9/64 C12Q1/37 A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	POTEMPA J ET AL: "THE INACTIVATION OF HUMAN PLASMA ALPHA-1 PROTEINASE INHIBITOR BY PROTEINASES FROM STAPHYLOCOCCUS-AUREUS" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 261, no. 30, 1986, pages 14330-14334, XP002154472 ISSN: 0021-9258 the whole document --- -/--	1, 2, 4-7, 29

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the international search

4 December 2000

Date of mailing of the international search report

21/12/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Van der Schaal, C



C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NELSON DANIEL ET AL: "Inactivation of alpha1-proteinase inhibitor as a broad screen for detecting proteolytic activities in unknown samples." ANALYTICAL BIOCHEMISTRY, vol. 260, no. 2, 1 July 1998 (1998-07-01), pages 230-236, XP002154473 ISSN: 0003-2697 cited in the application the whole document	1-26,29
X	--- S BUDAVARI ET AL: "The Merck Index" 1996 , MERCK & CO , WHITEHOUSE STATION XP002154475 item 5483 on page 932	30
P,X	--- NELSON DANIEL ET AL: "Purification and characterization of a novel cysteine proteinase (periodontain) from Porphyromonas gingivalis: Evidence for a role in the inactivation of human alpha1-proteinase inhibitor." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 18, 30 April 1999 (1999-04-30), pages 12245-12251, XP002154474 ISSN: 0021-9258 the whole document	1-30
P,X	--- WO 99 29870 A (AGIUS CATHERINE THERESE ;HOCKING DIANNA MARGARET (AU); MARGETTS MA) 17 June 1999 (1999-06-17) protein PG28, SEQ ID NO 432 -----	1-26,29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/10574

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9929870 A	17-06-1999	AU 1648799 A EP 1037999 A	28-06-1999 27-09-2000

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 235.00210201	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 00/ 10574	International filing date (day/month/year) 20/04/2000	(Earliest) Priority Date (day/month/year) 21/04/1999
Applicant THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.



It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.



the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :



contained in the international application in written form.



filed together with the international application in computer readable form.



furnished subsequently to this Authority in written form.



furnished subsequently to this Authority in computer readable form.



the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.



the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,



the text is approved as submitted by the applicant.



the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,



the text is approved as submitted by the applicant.



the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.



as suggested by the applicant.



because the applicant failed to suggest a figure.



because this figure better characterizes the invention.



None of the figures.

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING
OF A CHANGE(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

SANDBERG, Victoria, A.
Muetting, Raasch & Gebhardt, P.A.
P.O. Box 581415
Minneapolis, MN 55458-1415
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 22 February 2001 (22.02.01)	
Applicant's or agent's file reference 235.00210201	IMPORTANT NOTIFICATION
International application No. PCT/US00/10574	International filing date (day/month/year) 20 April 2000 (20.04.00)

1. The following indications appeared on record concerning: <input checked="" type="checkbox"/> the applicant <input checked="" type="checkbox"/> the inventor <input type="checkbox"/> the agent <input type="checkbox"/> the common representative		
Name and Address TRAVIS, James POTEMPA, Jan NELSON, Daniel	State of Nationality US	State of Residence US
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	
2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning: <input checked="" type="checkbox"/> the person <input type="checkbox"/> the name <input type="checkbox"/> the address <input type="checkbox"/> the nationality <input type="checkbox"/> the residence		
Name and Address	State of Nationality	State of Residence
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	
3. Further observations, if necessary: Please note that the status of the three applicant/inventors indicated above has changed from all designated States to US only.		
4. A copy of this notification has been sent to: <input checked="" type="checkbox"/> the receiving Office <input type="checkbox"/> the designated Offices concerned <input type="checkbox"/> the International Searching Authority <input checked="" type="checkbox"/> the elected Offices concerned <input checked="" type="checkbox"/> the International Preliminary Examining Authority <input type="checkbox"/> other:		

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Simin Baharlou Telephone No.: (41-22) 338.83.38
---	--

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE
in its capacity as elected Office

Date of mailing (day/month/year) 11 December 2000 (11.12.00)	
International application No. PCT/US00/10574	Applicant's or agent's file reference 235.00210201
International filing date (day/month/year) 20 April 2000 (20.04.00)	Priority date (day/month/year) 21 April 1999 (21.04.99)
Applicant TRAVIS, James et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
20 November 2000 (20.11.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Maria Kirchner Telephone No.: (41-22) 338.83.38
---	--

REC'D 19 JUL 2001


WIPO

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

14

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 235.00210201		FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/10574	International filing date (day/month/year) 20/04/2000	Priority date (day/month/year) 21/04/1999	
International Patent Classification (IPC) or national classification and IPC C12N15/57			
Applicant THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 6 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input type="checkbox"/> Certain observations on the international application 			
Date of submission of the demand 20/11/2000		Date of completion of this report 17.07.2001	
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465		Authorized officer Vix, O Telephone No. +49 89 2399 7326	



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/10574

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1-43 as originally filed

Claims, No.:

1-30 as originally filed

Drawings, sheets:

1/6-6/6 as originally filed

Sequence listing part of the description, pages:

1-13, filed with the letter of 03.11.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/US00/10574

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5,7,9,12-28
	No:	Claims	1-4,6,8,10-11,29-30
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-30
Industrial applicability (IA)	Yes:	Claims	1-30
	No:	Claims	

2. Citations and explanations
see separate sheet

Reference is made to the following documents:

- D1: POTEMPA J ET AL: 'THE INACTIVATION OF HUMAN PLASMA ALPHA-1 PROTEINASE INHIBITOR BY PROTEINASES FROM STAPHYLOCOCCUS-AUREUS' JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 261, no. 30, 1986, pages 14330-14334, ISSN: 0021-9258
- D2: S BUDAVARI ET AL: 'The Merck Index' 1996 , MERCK & CO , WHITEHOUSE STATION
- D3: WO 99 29870 A (AGIUS CATHERINE THERESE ;HOCKING DIANNA MARGARET (AU); MARGETTS MA) 17 June 1999 (1999-06-17)
- D4: NELSON DANIEL ET AL: 'Inactivation of alpha1-proteinase inhibitor as a broad screen for detecting proteolytic activities in unknown samples.' ANALYTICAL BIOCHEMISTRY, vol. 260, no. 2, 1 July 1998 (1998-07-01), pages 230-236,
- D5: NELSON DANIEL ET AL: 'Purification and characterization of a novel cysteine proteinase (periodontain) from Porphyromonas gingivalis: Evidence for a role in the inactivation of human alpha1-proteinase inhibitor.' JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 18, 30 April 1999 (1999-04-30), pages 12245-12251, ISSN: 0021-9258

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

- 1. Novelty (Art. 33(2) PCT)
 - 1.1 Priority documents have not been available at the time of establishing this preliminary opinion. The opinion has been established under the assumption of valid priority rights. Should this however not be the case, the document D4 and D5 cited in the ISR as P-documents might become important.
 - 1.2 Claim 1 relates to a bacterial polypeptide which has amidolytic activity for cleavage of a non-denaturated human α 1-proteinase inhibitor (α 1-PI) at a reactive site loop

region of the inhibitor. D1 clearly discloses the conversion, by three different proteinase (one of each is a cysteinyl proteinase), of the native human α 1-proteinase inhibitor into an inactive form due to the peptide bond cleavage near the reactive center of this protein. One of the enzyme used is a cysteinyl proteinase which was purified from *S. aureus* (see D1, Material and Methods). The human α 1-PI inactivation using cysteinyl proteinase was performed in a solution comprising Tris 100 mM at pH 8.0. The D1 analysis of the α 1-PI inactivation by the cystein proteinase (D1 table I) shows a cleavage site between the Glu and Ala residues of the α 1-PI reactive site loop (RSL).

Thus D1 is prejudicial to the novelty of claims 1-2, 4, 6 and 29.

- 1.3 D2 provides a screening method for detecting proteolytic activities based on the inactivation of α 1-PI RSL. This strategy was used to study culture supernatant of *P. gingivalis* which contains numerous cysteine proteinases (see D2 page 233). In particular, a new *P. gingivalis* cysteine proteinase was characterised and further purified: this enzyme appeared to inactivate α 1-PI and was inhibited by the cysteine proteinase inhibitor E-64 (but was unable to cleave peptide-pNA or proteins such as azocasein, collagen). The sequence of this new cysteine proteinase from *P. gingivalis* has not been disclosed in D2 and could not be compared to the one of the present application. Nevertheless, the characteristics of this new *P. gingivalis* enzyme is very closely related to the one presently claimed. Thus, the subject matter of the broad claims 1, 3-4, 8, 10 and 11 lacks novelty in view of D2.
- 1.4 A meaningful opinion with regard to novelty of claim 30 should not be established because it is not always possible to determine if any of the presently known substances is falling under the terms of said claim. Besides it is noted, that the compounds of claim 30 are not rendered novel just because of the fact that they have been identified by the method of claim 27, e.g. such compounds can already exist. Relating to this, several existing protease inhibitors are known to inhibit cysteinyl protease: thus, on the basis of D3 disclosing a class of protease inhibitors (leupeptins), the claim 30 is considered to lack novelty.

2. Inventive step (Art. 33(3) PCT)

The present application relates to an isolated polypeptide from *P. gingivalis* having amidolytic activity for cleavage of denaturated polypeptide and non denaturated serpin such as α 1-PI.

D2 is considered the closest prior art and describes screening method for detecting proteolytic activities based on the inactivation of α 1-PI RSL. As mentioned above, this strategy was used to study culture supernatant of *P. gingivalis* which contains numerous cysteine proteinases (see D2 page 233). In particular, a new *P. gingivalis* cysteine proteinase was characterised and further purified.

The sequence of this new cysteine proteinase from *P. gingivalis* has not been disclosed in D2 and could not be compared to the one of the present application. Nevertheless, the characteristics of this new *P. gingivalis* enzyme is very closely related to the one presently claimed. The D2 enzyme possessed the desired characteristics as it cleaved the α 1-PI RSL and was unable to cleave peptide-pNA or proteins such as azocasein, collagen.

The problem to be solved by the present invention may therefore be regarded as the provision of an alternative cysteine proteinase sequence isolated from *P. gingivalis*. The determination of such a polypeptide or polynucleotidic sequence is directly derivable from the teaching of D2 by using routine methods : D2 provides an assay to follow the isolation of this cysteine proteinase and its sequence determination merely involves routine methods for the skilled person in the art. Therefore, the sequence determination of the *P. gingivalis* proteinase does not include any step which would require an inventive activity.

Therefore, the subject-matter of claims 1-30 does not appear to involve an inventive step and does not satisfy the criterion set forth in Article 33(3) PCT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

MUETING, Ann M. et al
MUETING, RAASCH & GEBHARDT, P.A.
P.O. Box 581415
Minneapolis, MN 55458-1415
ETATS-UNIS D'AMERIQUE

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing
(day/month/year) 17.07.2001

Applicant's or agent's file reference
235.00210201

IMPORTANT NOTIFICATION

International application No.
PCT/US00/10574

International filing date (day/month/year)
20/04/2000

Priority date (day/month/year)
21/04/1999

Applicant
THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

RECEIVED

JUL 23 2001

MUETING & RAASCH

Name and mailing address of the IPEA/



European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer

Neumann, M

Tel. +49 89 2399-7351



PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 235.00210201	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/10574	International filing date (<i>day/month/year</i>) 20/04/2000	Priority date (<i>day/month/year</i>) 21/04/1999
International Patent Classification (IPC) or national classification and IPC C12N15/57		
Applicant THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 6 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 20/11/2000	Date of completion of this report 17.07.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Vix, O Telephone No. +49 89 2399 7326 

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/10574

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1-43 as originally filed

Claims, No.:

1-30 as originally filed

Drawings, sheets:

1/6-6/6 as originally filed

Sequence listing part of the description, pages:

1-13, filed with the letter of 03.11.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/10574

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5,7,9,12-28
	No:	Claims	1-4,6,8,10-11,29-30
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-30
Industrial applicability (IA)	Yes:	Claims	1-30
	No:	Claims	

2. Citations and explanations
see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/US00/10574

Reference is made to the following documents:

- D1: POTEMPA J ET AL: 'THE INACTIVATION OF HUMAN PLASMA ALPHA-1 PROTEINASE INHIBITOR BY PROTEINASES FROM STAPHYLOCOCCUS-AUREUS' JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 261, no. 30, 1986, pages 14330-14334, ISSN: 0021-9258
- D2: S BUDAVARI ET AL: 'The Merck Index' 1996 , MERCK & CO , WHITEHOUSE STATION
- D3: WO 99 29870 A (AGIUS CATHERINE THERESE ;HOCKING DIANNA MARGARET (AU); MARGETTS MA) 17 June 1999 (1999-06-17)
- D4: NELSON DANIEL ET AL: 'Inactivation of alpha1-proteinase inhibitor as a broad screen for detecting proteolytic activities in unknown samples.' ANALYTICAL BIOCHEMISTRY, vol. 260, no. 2, 1 July 1998 (1998-07-01), pages 230-236,
- D5: NELSON DANIEL ET AL: 'Purification and characterization of a novel cysteine proteinase (periodontain) from Porphyromonas gingivalis: Evidence for a role in the inactivation of human alpha1-proteinase inhibitor.' JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 18, 30 April 1999 (1999-04-30), pages 12245-12251, ISSN: 0021-9258

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

- 1. Novelty (Art. 33(2) PCT)
 - 1.1 Priority documents have not been available at the time of establishing this preliminary opinion. The opinion has been established under the assumption of valid priority rights. Should this however not be the case, the document D4 and D5 cited in the ISR as P-documents might become important.
 - 1.2 Claim 1 relates to a bacterial polypeptide which has amidolytic activity for cleavage of a non-denaturated human α 1-proteinase inhibitor (α 1-PI) at a reactive site loop

region of the inhibitor. D1 clearly discloses the conversion, by three different proteinase (one of each is a cysteinyl proteinase), of the native human α 1-proteinase inhibitor into an inactive form due to the peptide bond cleavage near the reactive center of this protein. One of the enzyme used is a cysteinyl proteinase which was purified from *S. aureus* (see D1, Material and Methods). The human α 1-PI inactivation using cysteinyl proteinase was performed in a solution comprising Tris 100 mM at pH 8.0. The D1 analysis of the α 1-PI inactivation by the cystein proteinase (D1 table I) shows a cleavage site between the Glu and Ala residues of the α 1-PI reactive site loop (RSL).

Thus D1 is prejudicial to the novelty of claims 1-2, 4, 6 and 29.

1.3 D2 provides a screening method for detecting proteolytic activities based on the inactivation of α 1-PI RSL. This strategy was used to study culture supernatant of *P. gingivalis* which contains numerous cysteine proteinases (see D2 page 233). In particular, a new *P. gingivalis* cysteine proteinase was characterised and further purified: this enzyme appeared to inactivate α 1-PI and was inhibited by the cysteine proteinase inhibitor E-64 (but was unable to cleave peptide-pNA or proteins such as azocasein, collagen). The sequence of this new cysteine proteinase from *P. gingivalis* has not been disclosed in D2 and could not be compared to the one of the present application. Nevertheless, the characteristics of this new *P. gingivalis* enzyme is very closely related to the one presently claimed. Thus, the subject matter of the broad claims 1, 3-4, 8, 10 and 11 lacks novelty in view of D2.

1.4 A meaningful opinion with regard to novelty of claim 30 should not be established because it is not always possible to determine if any of the presently known substances is falling under the terms of said claim. Besides it is noted, that the compounds of claim 30 are not rendered novel just because of the fact that they have been identified by the method of claim 27, e.g. such compounds can already exist. Relating to this, several existing protease inhibitors are known to inhibit cysteinyl protease: thus, on the basis of D3 disclosing a class of protease inhibitors (leupeptins), the claim 30 is considered to lack novelty.

2. Inventive step (Art. 33(3) PCT)

The present application relates to an isolated polypeptide from *P. gingivalis* having amidolytic activity for cleavage of denaturated polypeptide and non denaturated serpin such as α 1-P1.

D2 is considered the closest prior art and describes screening method for detecting proteolytic activities based on the inactivation of α 1-P1 RSL. As mentioned above, this strategy was used to study culture supernatant of *P. gingivalis* which contains numerous cysteine proteinases (see D2 page 233). In particular, a new *P. gingivalis* cysteine proteinase was characterised and further purified.

The sequence of this new cysteine proteinase from *P. gingivalis* has not been disclosed in D2 and could not be compared to the one of the present application. Nevertheless, the characteristics of this new *P. gingivalis* enzyme is very closely related to the one presently claimed. The D2 enzyme possessed the desired characteristics as it cleaved the α 1-P1 RSL and was unable to cleave peptide-pNA or proteins such as azocasein, collagen.

The problem to be solved by the present invention may therefore be regarded as the provision of an alternative cysteine proteinase sequence isolated from *P. gingivalis*. The determination of such a polypeptide or polynucleotidic sequence is directly derivable from the teaching of D2 by using routine methods : D2 provides an assay to follow the isolation of this cysteine proteinase and its sequence determination merely involves routine methods for the skilled person in the art. Therefore, the sequence determination of the *P. gingivalis* proteinase does not include any step which would require an inventive activity.

Therefore, the subject-matter of claims 1-30 does not appear to involve an inventive step and does not satisfy the criterion set forth in Article 33(3) PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/57, 9/64, C12Q 1/37, A61K 39/02	A2	(11) International Publication Number: WO 00/63394 (43) International Publication Date: 26 October 2000 (26.10.00)
(21) International Application Number: PCT/US00/10574 (22) International Filing Date: 20 April 2000 (20.04.00) (30) Priority Data: 60/130,436 21 April 1999 (21.04.99) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/130,436 (CON) Filed on 21 April 1999 (21.04.99) (71) Applicant (for all designated States except US): THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; 632 Boyd Graduate Studies, Athens, GA 30602-7411 (US). (71)(72) Applicants and Inventors: TRAVIS, James [US/US]; 825 Riverbend Parkway, Athens, GA 30605 (US). POTEPA, Jan [US/US]; Apartment #102, 170 Barrington Drive, Athens, GA 30605 (US). NELSON, Daniel [US/US]; Apartment 13-S, 504 East 63rd Street, New York, NY 10021 (US).		(74) Agent: SANDBERG, Victoria, A.; Mueting, Raasch & Gebhardt, P.A., P.O. Box 581415, Minneapolis, MN 55458-1415 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN (57) Abstract An isolated oral bacterial polypeptide having amidolytic activity for cleavage of denatured polypeptides and nondenatured serpin polypeptides and particularly a human α_1 -proteinase inhibitor polypeptide is provided. The mature polypeptide of the invention has a molecular weight of about 70 kD to about 80 kD. Also provided is an isolated nucleic acid sequence encoding the oral bacterial polypeptide of the invention, methods for identifying inhibitors of the polypeptide and compositions such as immunogenic compositions and inhibitor compositions.		

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DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

SEQ ID NO:

6 Streptopain 1 MNKKKLGRLSLLGLGGFVLNPVFAQNFARNEKEAKDSAIFIQKSAATKAGARSAE
 5 prtT 1 -MKKIFYTLGLDLLCLPML-QASPVTLSAEQTAKNFFAKROPTL----SSASRSD
 1 Periodontain 1 -MKKSELALFLFMQGHNPVTLSALSLA--LALROQLRMGOAVADKSD

Streptopain 61 DIKLDKVNLC----GELSGNMYVNIISTG-GFVVSQDKRSPEILGYSTSGSFDANGKE
 prtT 55 VYK--AAER-----EE--ALFEVNRGEKGFVLAADDRFPEVGYFKGFDAARF
 Periodontain 58 VYFQGD AERCITSOEEGSPAYFYVANRGNNFGALVAADDRIPITILNYSPIGFDMDSP

Streptopain 116 -----ISFMESYVEQIKENRLSTTYAGTAEIKOPVKSLLDSKGIENQO
 prtT 105 DNLRGWLKGYREILA MGKAEPIPIREAKPTRDPSSTAPSTGEHASDPILWQO
 Periodontain 118 DNLRMWLQIYQEILSGKACLN EIL---TEGPAETAAINCHFANDPWNQO

Streptopain 164 NPQNLTPLEKVKPGEQSFVGOHAATGCVATATAQIMYHNPNKGLKYTYTLLSNNP
 prtT 165 YPNTLHPLLP-----SGQAYTGC VATAMQIMRHYKWEKNSGYDYDDMTGT
 Periodontain 175 YPNNKEPLLP-----NGHAYTGC VATAAQIMRYHSWPLQGECS DYHAGSLVG

Streptopain 224 YFNHPKNL-----ISTROYNNWNNLPTYSGR-ESVOKMAYSELMDVGISVDMDPSSGS
 prtT 216 HT-----HSGTFGETYNWSKMPGNISG-SPEVKALSTEMRDVSFSVNMOFAFGSG
 Periodontain 226 N-----SGTFGE MYWINMPGNPDNDNSQVDAYTLMRDVSASVMSFYNGSC

Streptopain 283 AGSSRVORALENFGYNQSQINRSDKQWEAQDKELSONQPVYYQGVGVKGSHAF
 prtT 270 TSSVERALRETFYKKSLEYIHRSLLPGKEWDMIRKELAEENRPVYYAGADGSGHAF
 Periodontain 279 TSSVVGALRNFFYKKSLLQHLVRLLSQEWDMIRGELASGRPVYYAGNNQSGHAF

Streptopain 343 VIDGALGRNFHYNNGWGGVSNGFFALDALNPSALGTGGGAGGNGYQSAVGIKP----
 prtT 330 VCDGYPDGTFHFNWGWWGGSNGNFFYLLNLLNPGSLGTRGCGGTDQEVVIGIEPASNE
 Periodontain 339 VCDGYASDGTTFHFNWGWWGVSNGFLLTLLSPSLGIGGEIGFAYQETGIEPAKTP

prtT 390 VP-GIVDPDTILYGQHNMSDEA-LD-SVKIKNYSTYAGDKLYRLTLPNGTETTNPA
 Periodontain 399 AEAGTDALPIALKDTEAEYKSESGLNGYSIYNTGEEQSNDEYRLNKADGEVIEVKT

prtT 448 VPTVWEDIIGESTGNIIPCSQFAEGKNTISLYRTDGMADWKEPHILMGLVNKIEV
 Periodontain 459 SNNISWYG-YGEHPESFAPNQLSQGINTIILYRRTGTEQEPNHAQGGYVNSIKV

prtT 508 TMPAGDVAYSVA-----VVKDGSLSHDLKAYSDCKATVYNPGTEFRRVTFALRN
 Periodontain 518 NTTDPNNVVTVDNNESSVPNSFVADLNSYEHSTVQFNSDSPDIRTPVAFALST

prtT 565 --TEGRYFELGRHVEEHHPGDEDEGEKVSLLITGUKARAGQYMLVCTGDESLMEDASWIE
 Periodontain 578 GATADDISLGWVAEVPGGSSN-YPVWVKDVLTLSEGDTLWYRFSN--NQKDEWKK

prtT 623 SIEVAEHTSTHSSILVASNPQIDLLVHRANPETLPTFSSTNEGGATESCKEVAIK
 Periodontain 635 SSVKTPTEYTHPIFEVGHNOTSTYTDMAHNRVLPDEFKNLG-LPENGELVVFRO

prtT 683 AFSEI-FFOAKEEHSIAOGETKVLSEETENSSLYTNAELFPDGYIVYEQG-FWDP
 Periodontain 694 TQSSGSLWAAOETTHKOGETFYVKVVE-----PIPDGNYRATFAFVNGQQO

prtT 741 DLFGDYRYRTDLSSSDIAGKDVSTIVPNPAQYVHAIPPTYAGRLFDIQ
 Periodontain 745 FYLKGRNYTNGTAVEAE--SSEIRPNPAQDYVESAPCIPQELFDIS

prtT 801 GQVQSTKESDMRDVERLPKGTIIVVDMVGKIFIR-
 Periodontain 803 GQVVKNSISAHGRDVSRLPNCAYIKVQGYTKENIVH

Figure 1

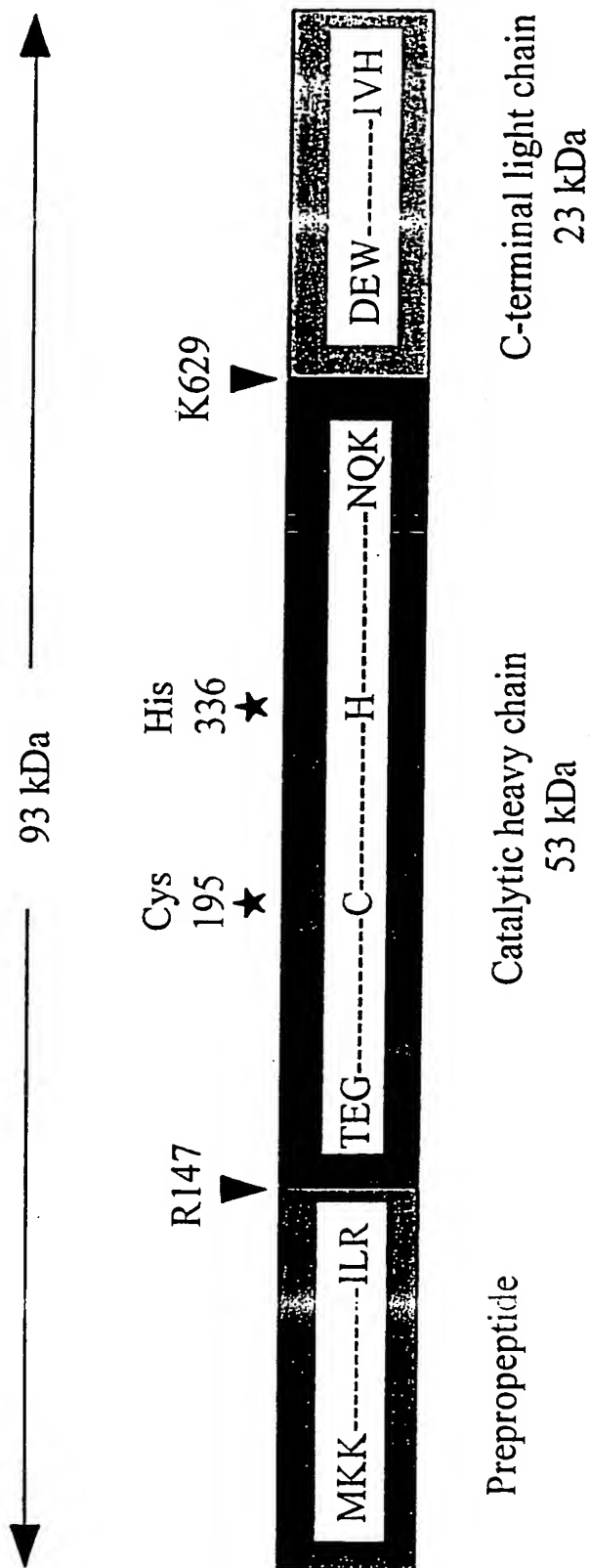


FIGURE 2

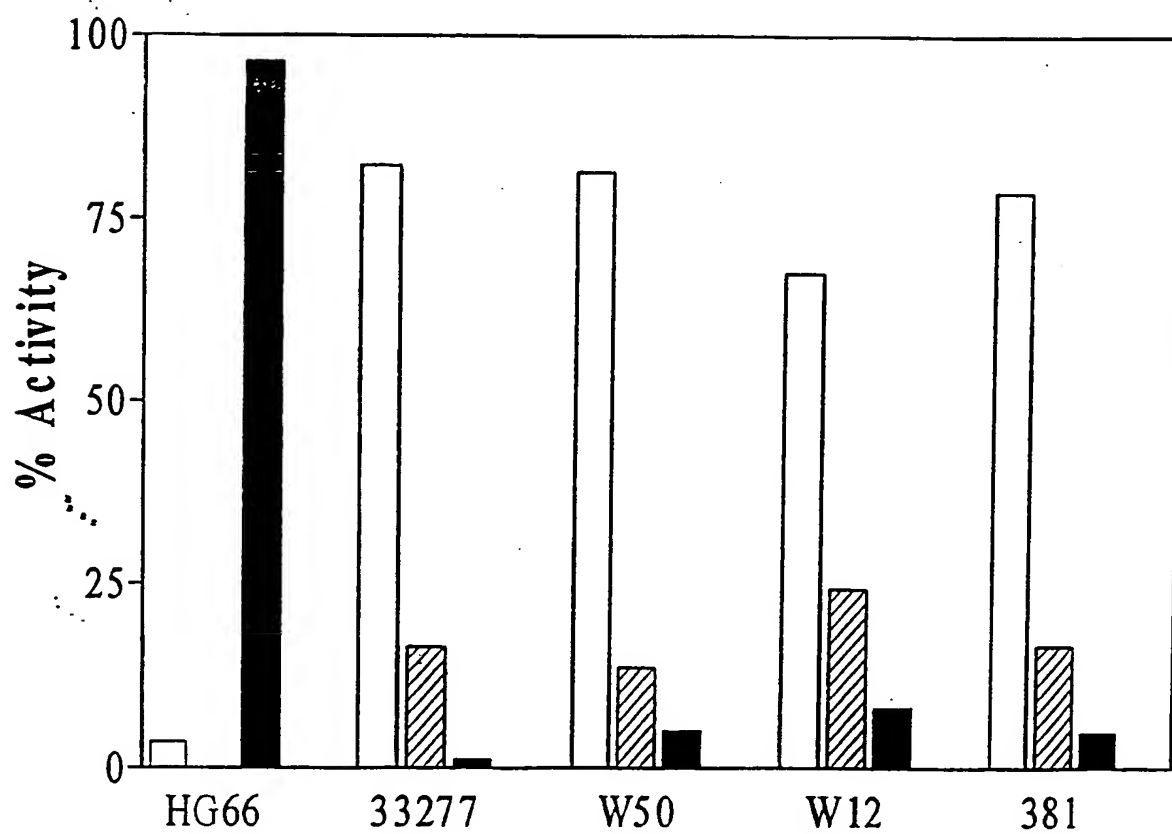


FIGURE 3

MPSSVSWGILLAGLCCLVPVSLAEDPQGDAQKTDTSHHQDHP
TFNKITPNLAFAFSLYRQLAHQSNSTNIFFSPVSIATAFAMLSLGTKADTHDEILEG
LNFNLTETPEAQIHEGFQELLRTLNPDSQLQLTTGNGLFLSEGLKLVOKFLEDVKKL
YHSEFTVNFGDHEEAKKQINDYVEKGTQGKIVDLVKELDRDTVFALVNYIFFKGKWE
RPFVVKDTEDEDFHVDQVTTVKVPMKRLGMFNIQHCKKLSSWVLLMKYLGNATAIFF
LPDEGKLOHLENELTHDIITKFLENEDRRSASLHLPKLSITGTDLKSVLGOLGITKV
FSNGADLSGVTEEAPLKLSKAVHKAVLTIDEKGTEAAGAMFLEAIPMSIPPEVKFNKP
FVFLMIEQNTKSPLFMGKVVNPTQK

SEQ ID No: 3

FIGURE 4.

ATGAAAAAAGTTTTCTTTTAGCCATAGTAATGCTCTTTGGCATTGCCATGCAGGGACAT
TCTGCTCCGGTTACGAAAGAGCGAGCTTTGAGTCTGGCTCGGCTGGCTTTGCGACAGGTA
TCCTTGCGAATGGGACAAACAGCAGTATCTGACAAGATTTCCATCGATTACGTTTATCGG
CAAGGAGATGCTGAGAGGGGTATCACATCACAAGAGGAAGGCTCTCCTGCATATTTTAT
GTAGCTAATCGTGGAAATAATGAGGGCTATGCTCTTGTAGCAGCAGATGACAGAATACCG
ACAATTTTAGCCTATTCACCCATTGGCCGTTTCGACATGGACAGTATGCCGAGCAATCTT
CGCATGTGGCTACAAATTTACGATCAGGAAATAGGCCTGATACTTTCCGGAAAAGCTCAG
CTCAATGAAGAGATATTACGTACCGAGGGCGTACCGGCTGAAGTACATGCTCTGATGGAT
AACGGTCATTTTGCCAACGATCCCATGCGATGGAATCAAGGTTACCCATGGAACAATAAG
GAACCACTGCTTCCTAATGGCAATCATGCCTATACCGGCTGTGTTGCTACTGCTGCAGCA
CAAATCATGCGCTACCATAGCTGGCCGCTTCAAGGTGAAGGCTCTTTCGATTATCATGCA
GGTTCATTAGTTGGCAACTGGTCCGGCACATTTGGTGAAATGTACGACTGGATCAATATG
CCCGGAAATCCCGACCTTGATAATCTGACTCAATCTCAAGTGGATGCCTACGCCACACTG
ATGCGTGATGTGAGTGCATCTGTTTCGATGAGTTTTTATGAAAATGGAAGTGGTACGTAC
AGCGTTTATGTAGTAGGAGCCTTGCGAAACAACCTTTCGCTACAAGCGTTCAGTGCAGCTA
CATGTACGCGCCTTATATACCTCACAGGAGTGGCACGATATGATCCGCGGGGAACCTGGC
TCCGGAAGGCCGGTCTATTATGCAGGGAATAACCAGAGCATAGGACATGCTTTCGTTTGC
GATGGTTATGCTTCGGATGGTACTTTCCATTTCAACTGGGGTTGGGGAGGTGTTTCCAAC
GGCTTCTACAAACTAACACTCCTCTCGCCGACTTCGTTGGGTATCGGAGGTGAGGGAATA
GGTTTTACCATTTATCAAGAGATCATCACCGGTATCGAACC GGCTAAGACTCCCGTGAA
GCCGGTACAGATGCCTTGCCGATCTTGGCACTGAAAGACATAGAAGCCGAGTATAAAAGT
GAA'TCCGGATTGAACGTAGGGTATTCGATATATAATACAGGTGAAGAGCAATCAAATCTT
GACCTCGGATACAGATTGAACAAGGCTGACGGAGAAGTCATAGAGGTGAA'AACTTCATCT
ATCAATATCTCTTGGTACGGATACGGAGAGCATCCCGAGAGTTTCTCATTGGCACCTAAT
CAGTTGTCACAAGGAATCAACACCATCACCTACTTTATCGTCGCACAGGCACCGAACAG
TGGGAGCCGGTACGGCATGCACAGGGAGGATATGTCAATAGCATTAAAGTAAATACGACA
GACCCGAACAATGTCGTAGTCACGGTAGATAATAACGAAGGCAAGCTCAGTATCGTCCCC
AACAGCTTTGTGCGCAGATCTGAATTCTTATGAACATAGTACGATTACAGTACAGTTCAAT
AGCGACAGCCCTGATGAGATCCGTACACCCGTAGCCTTTGCTCTATCTACAGGAGCTACT
GCGGACGATGTAATATCTTTGGGCTGGGTAATGGCTGAAGTTCGGGGCGGTAGCAGCAAC
TATCCGGTGGTTTGGTCTAAAGACGTTCTCACTCTCTCGGAAGGCGACTATACATTGTGG
TATAGATTTTCCATCAACAACCAAAAGGATGAATGGAAAAAGATCGGAAGCGTGTACAGTA
AAAACACCGACAGAGTATACGCACCCCTTATTCGAAGTGGGCCATAATCAAACCTTCTACC
TATACGCTGGATATGGCACACAACAGAGTATTGCCCGACTTTACACTCAAAAATCTCGGA
TTGCCTTTCAATGGTGAGTTGGTTGTTGTTTTCCGCCAAACACAATCCTCATCGGGTCT
TTATGGGCAGCTCAAGAAACAGTACATATCAAGCAAGGAGAACTTTTCGTATATAAACCT
GTTGTGGAAGGCCCTATACCTGATGGATCCTATCGTGCGACCTCCATGCATTGTAAC
GGACAACAACAGTTGTACCTCAAGGGGAAAAGGAACTACACGGTGAAGATCGTCAATGGT
ACAGCGGTAGAAGCAATAGAATCGTCAGAAGAGATCAGAGTATTCCTAATCCGGCACGC
GATTATGTGGAATATCGGCACCTTGCATTCCCCAAGAAACATCTATCATTCTTTTCGAT
CTGTACAGGCAAGATTGTCATGAAGAATAGTTTATCAGCGGGGCATGGCAGAATGGATGTC
AGCCGACTTCCTAATGGGGCCTACATCCTTAAGGTGGATGGATATACGACGAAAATAAAT
ATAGTGCATAA

SEQ ID No: 2

FIGURE 5.

GAMFLEAIPMSIPPE

SEQ ID NO: 4

FIGURE 6

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- (71) Applicant (for all designated States except US): THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; 632 Boyd Graduate Studies, Athens, GA 30602-7411 (US).
- (71) Applicants and
(72) Inventors: TRAVIS, James [US/US]; 825 Riverbend Parkway, Athens, GA 30605 (US). POTEPA, Jan [US/US]; Apartment #102, 170 Barrington Drive, Athens, GA 30605 (US). NELSON, Daniel [US/US]; Apartment 13-S, 504 East 63rd Street, New York, NY 10021 (US).
- (74) Agent: SANDBERG, Victoria, A.; Muetting, Raasch & Gebhardt, P.A., P.O. Box 581415, Minneapolis, MN 55458-1415 (US).
- (81) Designated States (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
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(54) Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

(57) Abstract: An isolated oral bacterial polypeptide having amidolytic activity for cleavage of denatured polypeptides and non-denatured serpin polypeptides and particularly a human α_1 -proteinase inhibitor polypeptide is provided. The mature polypeptide of the invention has a molecular weight of about 70 kD to about 80 kD. Also provided is an isolated nucleic acid sequence encoding the oral bacterial polypeptide of the invention, methods for identifying inhibitors of the polypeptide and compositions such as immunogenic compositions and inhibitor compositions.

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INTERNATIONAL SEARCH REPORT

International Application No

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A. CLASSIFICATION OF SUBJECT MATTER

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	POTEMPA J ET AL: "THE INACTIVATION OF HUMAN PLASMA ALPHA-1 PROTEINASE INHIBITOR BY PROTEINASES FROM STAPHYLOCOCCUS-AUREUS" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 261, no. 30, 1986, pages 14330-14334, XP002154472 ISSN: 0021-9258 the whole document --- -/--	1, 2, 4-7, 29



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

4 December 2000

Date of mailing of the international search report

21/12/2000

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European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Van der Schaal, C

INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NELSON DANIEL ET AL: "Inactivation of alpha1-proteinase inhibitor as a broad screen for detecting proteolytic activities in unknown samples." ANALYTICAL BIOCHEMISTRY, vol. 260, no. 2, 1 July 1998 (1998-07-01), pages 230-236, XP002154473 ISSN: 0003-2697 cited in the application the whole document	1-26,29
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INTERNATIONAL SEARCH REPORT

information on patent family members

Inter. Appl. Application No

PCT/US 00/10574

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		EP 1037999 A	27-09-2000

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- (71) Applicant (*for all designated States except US*): THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; 632 Boyd Graduate Studies, Athens, GA 30602-7411 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): TRAVIS, James [US/US]; 825 Riverbend Parkway, Athens, GA 30605 (US). POTEPA, Jan [US/US]; Apartment #102, 170 Barrington Drive, Athens, GA 30605 (US). NELSON, Daniel [US/US]; Apartment 13-S, 504 East 63rd Street, New York, NY 10021 (US).
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(54) Title: A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

(57) Abstract: An isolated oral bacterial polypeptide having amidolytic activity for cleavage of denatured polypeptides and non-denatured serpin polypeptides and particularly a human α_1 -proteinase inhibitor polypeptide is provided. The mature polypeptide of the invention has a molecular weight of about 70 kD to about 80 kD. Also provided is an isolated nucleic acid sequence encoding the oral bacterial polypeptide of the invention, methods for identifying inhibitors of the polypeptide and compositions such as immunogenic compositions and inhibitor compositions.

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A POLYPEPTIDE HAVING AMIDOLYTIC ACTIVITY FOR A SERPIN

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Statement of Government Rights

The present invention was made with support from the National Institutes of Health under Grant No. DE09761. The government may have certain rights in the invention.

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Background

The anaerobe *Porphyromonas gingivalis* (*P. gingivalis*) has been implicated as a major causative organism of adult onset periodontal disease. Enzymes from this organism have been found to degrade several proteins, including, for example, collagen, fibrinogen, immunoglobulins, complement proteins, and fibronectin. Recent evidence has shown that three proteinases released from *P. gingivalis* may have a physiological role in modulating the human immune system in addition to their general ability to degrade proteins. These three proteinases are referred to as gingipains and include arginine-specific gingipain A (RgpA) and arginine-specific gingipain B (Rgp B), which are capable of specifically cleaving after arginine residues, and lysine-specific gingipain (Kgp) which is able to specifically cleave after lysine residues. Working in concert, these proteinases have been shown to produce bradykinin from high molecular weight kininogen, either directly or indirectly (kallikrein activation), resulting in the enhancement of vascular permeability (Imamura, et al., Infect. Immun., 63(5):1999-2003 (1995)). This mechanism, which is used to provide nutritional components for the growth and proliferation of *P. gingivalis*, is presumed to be responsible for both the increased gingival crevicular fluid (GCF) and edema clinically noted in periodontal pockets of patients with advanced periodontitis (Darany et al., J. Periodontol., 63:743-747 (1992)).

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The interaction of *P. gingivalis* with a host's immune system response has been paradoxical, in that *P. gingivalis* has demonstrated both pro-

inflammatory and anti-inflammatory responses. For example, *P. gingivalis* lipopolysaccharide has been shown to increase mRNA levels of interleukin-8 (IL-8) in neutrophils (Sugita et al., Inflammation, 22(3):253-267(1998)), and gingipains R have been shown to increase neutrophil chemotaxis by release of
5 C5a from C5 of the complement system (Wingrove et al., J. Biol. Chem., 267(26):18902-18907 (1992)). However, these proteinases are also capable of cleaving the C5a receptor from infiltrating neutrophils (Jagels et al., Infect. Immun., 64(6):1984-1991 (1996)), thereby effectively neutralizing localized chemotactic activity. Additionally, *P. gingivalis* has the ability to inhibit both
10 IL-8 accumulation in gingival epithelial cells (Darveau et al., Infect. Immun., 66(4):1660-1665 (1998)), as well as transepithelial migration (Madianos et al., Infect. Immun., 65(10):3983-3990 (1997)).

These apparent activity contradictions may potentially be explained by the compartmentalization of the oral cavity, wherein distal activation of
15 chemotactic components and proximal paralysis of these factors creates a "leukocyte wall" between the periodontal plaque and gingival epithelium (Miyasaki, J. Periodontol., 62:761-774 (1991)). Indeed, it has been reported that soluble gingipains can stimulate IL-8 activity, whereas membrane bound gingipains, with a limited ability to diffuse beyond the plaque surface,
20 completely degrade IL-8 (Mikolajczyk-Pawlinska et al., FEBS Lett., 440:282-286 (1998)).

The recruitment of neutrophils to the "leukocyte wall" through both the increased leakage of blood vessels and a chemotactic gradient at first appear to be suicidal to *P. gingivalis*. However, such a scenario is not likely, as *P.*
25 *gingivalis* is known to have evolved mechanisms to survive in the presence of neutrophils. For example, *P. gingivalis* proteinases have been shown to degrade C3 complement and immunoglobulins (Schenkein et al., J. Immunol., 154:5331-5337 (1995)), thereby averting opsonization and subsequent detection by a host. Furthermore, gingipain R has been shown to have an inhibitory effect on the
30 oxidative burst utilized by neutrophils to kill microorganisms (Kadowaki et al., J. Biol. Chem., 269(33):21371-21378 (1994)). Similarly, the bacterial outer membrane of *P. gingivalis* may function as an antioxidant sink due to the

incorporation of large amounts of heme (Smalley et al., *J. Biochem.*, **331**:681-685 (1998)).

Activated neutrophils in the leukocyte wall typically undergo degranulation due to the inability to phagocytize foreign organisms, thereby expelling large quantities of the proteinases human neutrophil elastase (HNE) and cathepsin G. Although these proteinases may cause abnormal connective tissue destruction, the presence of human plasma proteinase inhibitors (serpins) typically minimize connective tissue destruction by complexing with endogenous proteinases. These complexes are ultimately absorbed by the liver for degradation. For example, high protein levels of the α_1 -proteinase inhibitor (α_1 -PI) have been detected in GCF samples from patients diagnosed with severe periodontal disease (Huynh et al., *J. Clin. Periodontol.*, **19**:187-192 (1992)). However, despite the presence of α_1 -PI, a high HNE activity is observed indicating that the α_1 -PI must be present in either complexed, oxidized, or proteolytically inactivated forms (Uitto et al., *J. Clin. Periodontol.*, **23**:30-37 (1996)). This observation is supported by evidence showing that less than 35% of available α_1 -PI in the GCF is active as an inhibitor (Smith et al., *Archs. Oral Biol.*, **39**(4):301-306 (1994)). Additionally, it has been shown that patients with α_1 -PI deficiencies have a significantly higher frequency of periodontal pocket depths ≥ 5 mm, thereby being predisposed to manifestations of periodontal disease (Fokkema et al., *J. Clin. Periodontol.*, **25**:617-623 (1998)). Thus, there is a need for further understanding the interaction of such serpins with other oral bacterial proteinases.

Summary of the Invention

Described herein are the isolation, purification and characterization of a polypeptide, particularly an oral bacterial polypeptide that interacts with a serpin such as the human serpin α_1 -PI. This polypeptide is referred to as "periodontain," not only because of its function as a proteinase, but also because this polypeptide may function as a putative factor in the dysregulation of serpin function in the periodontal cavity of an animal. Additionally, the deduced amino

acid sequence of periodontain, as determined by both partial peptide sequencing of the purified polypeptide and characterization of the *P. gingivalis* genome is provided.

Accordingly, the present invention provides an isolated oral bacterial polypeptide which has amidolytic activity for cleavage of a nondenatured human α_1 -proteinase inhibitor at a reactive site loop region of the inhibitor. The isolated polypeptide demonstrates amidolytic activity in a solution containing about 1 mM to about 500 mM Tris, about 500 μ M to about 100 mM cysteine maintained at a pH of about 7 to about 8. Preferably, the polypeptide is isolated from *Porphyromonas gingivalis* and is a cysteine proteinase.

The polypeptide of the invention preferably has a molecular weight of about 70 kD to about 80 kD as determined by gel filtration. The polypeptide of the invention will preferably cleave the reactive site loop region of the inhibitor represented by SEQ ID NO: 4 between glutamine and alanine and also between phenylalanine and leucine.

The present invention also provides an isolated polypeptide that is an oral bacterial cysteine proteinase and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin. The isolated polypeptide is preferably isolated from *Porphyromonas gingivalis*. The isolated polypeptide of the invention further has the capability to cleave a target polypeptide nonspecifically.

The present invention further provides an isolated polypeptide that is isolated from *Porphyromonas gingivalis* and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.

The isolated polypeptide preferably contains an amino acid sequence having a percentage amino acid identity of greater than 37% to that of amino acid 148 to amino acid 843 of SEQ ID NO: 1. More preferably, the isolated polypeptide contains an amino acid sequence having a percentage amino acid identity of greater than 52% to that of amino acid 148 to amino acid 629 of SEQ ID NO: 1. In particularly preferred embodiments, the isolated polypeptide has an amino acid sequence represented by SEQ ID NO: 1, an active analog or an active fragment thereof, and more preferably, an amino acid sequence

represented by amino acid 148 to amino acid 843 of SEQ ID NO: 1, an active analog or an active fragment thereof.

The present invention further provides an isolated nucleic acid encoding an oral bacterial polypeptide which has amidolytic activity for cleavage of a
5 nondenatured human α_1 -proteinase inhibitor at a reactive site loop region of the inhibitor. Also provided is an isolated nucleic acid encoding a polypeptide which is an oral bacterial cysteine proteinase and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin. The present invention further provides an isolated nucleic acid encoding a
10 polypeptide which is isolated from *Porphyromonas gingivalis* and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.

The isolated nucleic acid fragment of the invention preferably has a nucleotide sequence represented by SEQ ID NO: 2. The isolated nucleic acid
15 fragment of the invention further preferably encodes a polypeptide having an amino acid sequence with a percentage amino acid identity of greater than 37% when compared to amino acid 148 to amino acid 843 of SEQ ID NO: 1.

The present invention also provides an isolated nucleic acid fragment as described herein wherein the complement of the nucleic acid fragment
20 hybridizes to SEQ ID NO: 2 under hybridization conditions of 0.5 M phosphate buffer, pH 7.2, 7% sodium dodecyl sulfate (SDS), 10 mM ethylenediaminetetraacetate (EDTA), at 68° C, followed by three 20 minute washes in 2x SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, at 65° C, wherein at least about 15 nucleotides of the complement hybridize.

25 The present invention further provides a method for identifying an inhibitor of a polypeptide which has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin. The method includes isolating an agent that inhibits the amidolytic activity of the polypeptide by incubating the polypeptide with the agent under conditions that promote
30 amidolytic activity of the polypeptide and determining if the amidolytic activity of the polypeptide is reduced relative to the amidolytic activity of the

polypeptide in the absence of the agent. Preferably, the polypeptide is isolated from *Porphyromonas gingivalis*.

The invention also provides an immunogenic composition having a polypeptide which has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin and is capable of eliciting antibodies in an animal.

Also provided is a composition containing an inhibitor to a polypeptide that has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin isolated from an oral bacterium.

10

Definitions

"Polypeptide" as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, and enzyme are included within the definition of polypeptide. This term also includes post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, and the like.

"Proteinase," "peptidase," and "protease" all refer to enzymes that catalyze the hydrolysis of peptide bonds in a polypeptide. A "peptide bond" or "amide bond" is a covalent bond between the alpha-amino group of one amino acid and the alpha-carboxyl group of another amino acid.

As used herein, the term "isolated" means that a polypeptide is either removed from its natural environment or synthetically derived. Preferably, the polypeptide is purified, i.e., essentially free from any other polypeptides and associated cellular products or other impurities.

"Amidolytic activity" refers to the ability of a polypeptide to catalyze the hydrolysis of at least one peptide bond in another polypeptide. The term "cleavage" can also be used to refer to the hydrolysis of a peptide bond in a polypeptide.

"Periodontain" refers to a polypeptide having amidolytic activity with respect to a target polypeptide. Preferably, the polypeptide is a cysteine proteinase that is isolated from an oral bacteria, such as, *P. gingivalis*. A "target

polypeptide,” as used herein, includes any denatured polypeptide and/or a native polypeptide, i.e., nondenatured, that contains at least one exposed contiguous amino acid region, such as a random coil as determined for example, by x-ray crystallography, that is susceptible to cleavage, preferably by periodontain. The exposed amino acid region typically contains at least about 3 contiguous amino acids, and preferably at least about 10 contiguous amino acids. Periodontain target polypeptides include, for example, serpin polypeptides in general, and mammalian serpin polypeptides specifically. A preferred target polypeptide of the invention is the human serpin, α_1 -proteinase inhibitor (α_1 -PI) (SEQ ID NO: 3). With respect to a serpin polypeptide, the exposed amino acid region, e.g., random coil, as used herein, is referred to as a “reactive site loop” region or “RSL.” The RSL region of human α_1 -PI is about 15 contiguous amino acids (SEQ ID NO: 4).

An “active analog” or “active fragment” of a polypeptide of the invention is one that has amidolytic activity by hydrolysis of a peptide bond present in the target polypeptide as described herein. Active analogs and active fragments are described in greater detail herein.

“Nucleic acid fragment” as used herein refers to a linear polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double- and single-stranded DNA and RNA. A nucleic acid fragment may include both coding and noncoding regions that can be obtained directly from a natural source (e.g., a microorganism), or can be prepared with the aid of recombinant or synthetic techniques. A nucleic acid molecule may be equivalent to this nucleic acid fragment or it can include this fragment in addition to one or more other nucleotides or polynucleotides. For example, the nucleic acid molecule of the invention can be a vector, such as an expression or cloning vector.

“Percentage amino acid identity” refers to a comparison of the amino acids of two polypeptides as described herein. Amino acid alignment may be determined, for example, using the sequence alignment program CLUSTAL W available at www.genome.ad.jp/SIT/CLUSTALW.html, and percent amino acid

identity may be determined by BLAST 2 SEQUENCES at National Center for Biotechnology Information (NCBI) website: www.ncbi.nlm.nih.gov.

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Brief Description of the Drawings

Figure 1. Sequence alignment of periodontain, *PrtT*, and streptopain.

The aligned putative gene products of *prtT* (SEQ ID NO: 5) and periodontain (SEQ ID NO: 1), both deduced from *P. gingivalis* strain W83 genome, and streptopain (SEQ ID NO: 6), from *S. pyogenes*, were aligned according to multiple sequence alignment using the sequence alignment program CLUSTAL W and the percent amino acid identity was determined by BLAST 2 SEQUENCES at NCBI using the default matrix. The black box(es) indicate that the same amino acid is present in a compared sequence as indicated, and grey box(es) indicate that a similar amino acid, e.g., a conservative amino acid substitution, is present in a compared sequence as indicated. The putative catalytic cysteine and histidine residues of streptopain are marked (*). The underlined residues indicate the obtained N-terminal sequence of the light chain of periodontain. The double underlined residues indicate the sequence obtained from both N-terminal sequencing of the heavy chain and DNA sequencing of the 69 basepair PCR product. Arrows indicate the putative cleavage sites necessary to form a mature periodontain heterodimer polypeptide from a nascent polypeptide.

25 Figure 2. Proposed processing for the maturation of periodontain.

Structure of the periodontain polypeptide with proposed processing sites based on N-terminal sequencing of the purified heterodimer.

Figure 3. Distribution of peri dontain activity in various *P. gingivalis* strains.

Periodontain activity from the indicated strains measured against human α_1 -PI using the assay as described in Material and Methods. Cell associated
5 activities (clear bar), vesicle associated activities (hatched bar), and soluble activities (solid bar) were normalized to 100% for each strain.

Figure 4. The human α_1 -proteinase inhibitor amino acid sequence.

The amino acid sequence of human α_1 -PI is shown. The RSL region of
10 human α_1 -PI is identified.

Figure 5. The nucleic acid sequence of periodontain.

The nucleic acid sequence for the full-length nascent polypeptide of the
invention is shown.

15

Figure 6. The RSL region of human α_1 -PI.

Detailed Description of Preferred Embodiments

Periodontal disease in an animal host, is typically characterized by
20 inflammation of the periodontium manifested by recruitment of neutrophils which can degranulate, releasing powerful proteinases responsible for destruction of connective tissues, and eventual loss of tooth attachment in the animal host. Although the presence of a host's innate proteinase inhibitors (i.e., serpins) may minimize tissue damage by endogenous proteinases, this is not
25 clinically observed, and it has been speculated that proteolytic inactivation of a host's proteinase inhibitors may contribute to progression of the disease. A major pathogen associated with periodontal disease in an animal is the gram negative oral bacteria, *Porphyromonas gingivalis*. "Animal," as used herein, includes, for example, mammals, such as humans, sheep and dogs.

30 Described herein is a preferred polypeptide that has been isolated from the culture supernatants of *Porphyromonas gingivalis*. The polypeptide is capable of inactivating, for example, the nondenatured human serpin α_1 -

proteinase inhibitor (α_1 -PI), which is the primary endogenous regulator of human neutrophil elastase (HNE). A preferred polypeptide, referred to herein as periodontain, belongs to the cysteine proteinase family, and as a mature polypeptide, exists as a heterodimer. Periodontain (SEQ ID NO: 1) shares
5 limited amino acid sequence identity to streptopain (SEQ ID NO: 6), a proteinase from *Streptococcus pyogenes*, and *prtT* (SEQ ID NO: 5), a putative proteinase from *P. gingivalis* (Table III and Figure 1). Thus, a preferred polypeptide of the invention typically has a percentage amino acid identity of greater than 37% of the amino acid sequence including amino acid 148 to amino
10 acid 843 of SEQ ID NO: 1. More preferably, the polypeptide of the invention has a percentage amino acid identity of greater than about 52%, even more preferably greater than about 70%, and most preferably greater than about 85% compared to the amino acid sequence of SEQ ID NO: 1.

Serpins, such as human α_1 -PI, are single chain proteins containing a
15 conserved domain structure of about 370 to about 390 residues, usually flanked by amino- or carboxyl- terminal extensions. Those present in plasma are also variably glycosylated, although the carbohydrate side chains are not required for activity. Inhibitory serpins interact with their target proteinase at a reactive site located within a loop structure 30 to 40 amino acids from the carboxyl terminus.
20 This area is exposed on the surface of the protein and is susceptible to proteolysis by nontarget proteinases. See, for example, Potempa et al., J. Biol. Chem., 269:15957-15960 (1994).

The primary function of members of the serpin family is to neutralize overexpressed serine proteinase activity. This involves highly specific binding
25 with a target enzyme by complex formation at a pseudo-reactive site peptide bond or "bait region" of the inhibitor. For example, the bait region in human α_1 -PI is the peptide bond between methionine (amino acid residue 358 of SEQ ID NO: 3) and serine (amino acid residue 359 of SEQ ID NO: 3). In the case of human α_1 -PI, the fidelity of this interaction is primarily dictated by the structure
30 of the methionine residue which is usually in agreement with the cleavage specificity of the proteinase being inactivated. It has been shown that oxidation

of human α_1 -PI converts the methionine amino acid residue to "Met-SO" (Travis et al., Annu. Rev. Biochem., 52:655-709 (1983)). Concomitant with this reaction is a dramatic loss of functional activity. It is now believed that, *in vivo*, this may be a mechanism to regulate elastase inhibitory activity at inflammatory sites. It is believed that the presence of periodontain *in vivo*, could result in elevated levels of HNE clinically detected in periodontal disease and may be a potential virulence factor for *P. gingivalis*. Although it was recently demonstrated that *P. gingivalis* produces a proteinase that rapidly inactivates α_1 -PI, the proteinase was not isolated (Nelson et al., Anal. Biochem., 260:230-236 (1998)) until now.

The present invention provides an isolated polypeptide, preferably an isolated proteinase, and more preferably a cysteine proteinase, that has amidolytic activity as evidenced by the hydrolysis of a peptide bond present in a target polypeptide. In general, the amidolytic activity of the polypeptide can be measured by assay of the cleavage of a target polypeptide in the presence of the polypeptide and a buffer (see, for example, Example 1). Preferably, the polypeptide is isolated from an oral bacteria. "Oral," as used herein, refers to biological material, such as bacteria, that can be found and isolated from the mouth of an animal.

In its nascent form, the isolated polypeptide of the invention contains about 843 amino acid residues and has a molecular weight of about 93 kD (Figure 1). "Nascent," as used herein, refers to a full-length unprocessed 93 kD polypeptide containing amino acid 1 to amino acid 843 of SEQ ID NO: 1 and as shown in Figure 1. The 93 kD polypeptide is unprocessed in that it contains additional amino acids not present in the mature polypeptide of the invention.

As shown in Figure 2, proteolytic processing of the nascent 93 kD polypeptide at amino acid 147 (Arg) and amino acid 629 (Lys) yields a prepro-polypeptide (amino acids 1-147), a heavy chain polypeptide containing a catalytic region (amino acids 148-629), and a light chain polypeptide containing the C-terminal portion of the polypeptide corresponding to amino acid 630 to amino acid 843. As used herein, the "catalytic region" or "catalytic subunit" of the heavy chain polypeptide of periodontain (or any polypeptide described

herein) is a region that is able to cleave, i.e., hydrolyze, a target polypeptide, particularly a nondenatured serpin. For example, in the RSL region of non-denatured human serpin α_1 -PI, periodontain will specifically cleave the peptide bond between glutamic acid and alanine residues (amino acids 354 and 355, respectively, of SEQ ID NO: 3). Typically, cleavage of this peptide bond is observed *in vitro* in a solution containing a Tris buffer in a concentration of about 1 millimolar (mM) to about 500 mM, about a 500 micromolar (μ M) to about 100 mM cysteine at a pH of about 7 to about 8 and a temperature of about 18° C to about 42° C. Preferably the solution contains about 50 mM Tris, about 20 mM cysteine maintained at a pH of about 7.4. Although cleavage of the peptide bond may occur at ambient temperature (typically about 25° C to about 30° C), preferably a temperature of about 37°C is employed. Periodontain will also specifically cleave the peptide bond between phenylalanine and leucine residues (amino acids 352 and 353, respectively of SEQ ID NO: 3) under the solution conditions set forth above. Cleavage of this latter peptide bond occurs at about half the catalytic rate as cleavage of the peptide bond between the glutamic acid and alanine residues described above.

Without being bound by any specific theory, periodontain and other polypeptides of the invention are additionally characterized by the nonspecific proteolysis of a target polypeptide in that a specific amino acid residue residing in the polypeptide does not necessarily dictate the specificity of the proteinase. For example, periodontain will cleave any target polypeptide nonspecifically in a denaturing environment. A "denaturing environment," as used herein, includes *in vitro* environment conditions, for example, heat, strong reducing agents, such as dithiothreitol and β -mercaptoethanol, sodium dodecyl sulfate, or other chemical manipulation including carboxymethylate-maleylate treatment. A "denaturing environment" also includes *in vivo* denaturing environment conditions, such *in vivo* denaturing environment conditions may be provided by the catalytic action of other *in vivo* polypeptides, for example, gingipain A and gingipain B.

A "mature polypeptide" of the invention is a heterodimer and contains the heavy polypeptide chain, the light polypeptide chain and has proteolytic

enzymatic activity. The polypeptide is mature in that it lacks the prepro region of the nascent polypeptide, e.g., amino acid 1 to amino acid 147 or SEQ ID NO: 1, and is not associated with a tRNA. The mature polypeptide has a predicted molecular weight of about 70 kD to about 80 kD, and preferably a molecular weight of about 75 kD to about 77 kD as determined by gel filtration. The heavy polypeptide chain typically has a molecular weight of about 50 kD to about 57 kD, and preferably a molecular weight of about 52 kD to about 55 kD as determined by gel filtration. The heavy chain preferably contains the catalytic site of the polypeptide. The mature polypeptide also contains the C-terminal light chain having a molecular weight of about 20 kD to about 25 kD, preferably a molecular weight of about 22 kD to about 24 kD. The C-terminal light chain appears to be devoid of enzymatic activity. Additionally, the mature polypeptide has an isoelectric focusing point (pI) of about 5.1 to about 5.5.

The *in vivo* activity of periodontain may have important physiological significance, as it may lead to the inactivation of many biologically active polypeptides and/or transformation of the activity of other biologically active polypeptides. In addition, hydrolysis of peptide bonds by periodontain in conjunction with general catabolic pathways should allow the complete re-utilization of amino acids by living organisms, including bacteria. However, proteinases from bacterial pathogens, if released into the host environment, may interfere with the physiological functions of biologically active polypeptides and, therefore, contribute to the pathogenicity of infectious disease.

The external (i.e., cell surface) localization and uncontrolled activity of a bacterial proteinase, including periodontain, likely contributes significantly to run-away inflammation in the animal host and the pathological degradation of connective tissue during periodontitis. Periodontain, although unable to degrade proteins such as azocasein, casein, lysozyme, collagen, fibrin, plasminogen, and fibrinogen in nature, may be capable of degrading these proteins *in vitro* if present in a denaturing environment as described above. For example, when lysozyme is denatured, i.e., carboxymethylated-maleylated lysozyme, complete digestion of the reduced form of lysozyme by periodontain rapidly occurs. This characteristic indicates that periodontain will readily cleave denatured or easily

accessible polypeptides, but is typically unable to cleave some polypeptides having defined secondary or tertiary structure, such as the native proteins indicated above; human α_1 -PI being the exception.

Based on the requirement that a denaturing environment is typically
5 needed for periodontain to actively cleave a target polypeptide, periodontain has been classified as a cysteine proteinase. This observation is further confirmed by the fact that periodontain is readily inhibited by common cysteine proteinase inhibitors selected from the group consisting of dichloroisocoumarin, diisopropylfluorophosphate, leupeptin, tosyl-L-lysine chloromethyl ketone, Phe-
10 Pro-Arg chloromethyl ketone, Z-Phe-Lys benzoyloxy methyl ketone, idoacetamide and L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64). The ability of E-64 to inhibit periodontain further suggests that periodontain is more closely related to members of the papain family of enzymes than other cysteine proteinases of *P. gingivalis*.

15 The polypeptide of the present invention, preferably a cysteine proteinase, can be used as a source of inhibitors (in addition to those described above), such as inhibitory polypeptides and antibodies, for inhibiting the peptidase activity and thereby possibly reducing periodontitis, loss of tooth attachment and periodontal pocket formation. Antibodies to the cysteine
20 proteinase of the invention can also be used to identify and/or isolate additional cysteine proteinases. Knowledge of specific cysteine proteinases can also be used to make inhibitory polypeptides against other cysteine proteinases. Such inhibitory polypeptides typically include from about 2 to about 20 amino acids from a polypeptide that the cysteine proteinase has at least some specificity for.
25 such as the specificity that periodontain has for human α_1 -PI, further associated with a chemical group that inactivates the active site of the proteinase. such as chloromethyl ketone and organo-phosphinate compounds. These inhibitory polypeptides and antibodies may be employed to possibly reduce gingivitis, periodontitis, loss of tooth attachment, and/or periodontal pocket formation.

30 As described herein, an example of a specific cysteine proteinase is the cysteine proteinase periodontain isolated from *P. gingivalis* (SEQ ID NO: 1). Periodontain is a unique proteolytic enzyme, and the isolation and

characterization of this novel bacterial proteinase will facilitate the development of therapies that function to inhibit the activity of this and other bacterial proteinases. Although not intending to be limiting, it is conceivable that periodontain is membrane anchored through a putative signal sequence which is not cleaved but remains as a membrane spanning domain similar to other members of the papain family of cysteine proteinases. Periodontain is most frequently associated with the membrane and outer membrane vesicles in strains 2561 (ATCC Accession No: 33277, Bethesda, MD), W50 (ATCC Accession No: 53978, Bethesda, MD), W12, and 381, despite the fact that it is soluble in strain HG66 (Figure 3). This is also in agreement with the distribution of other *P. gingivalis* proteinases.

A preferred cysteine proteinase of the invention, is the mature polypeptide containing amino acid 148 to amino acid 843 of SEQ ID NO: 1. As shown in Figure 1, it is thought that the catalytic domain of periodontain corresponds to the cysteine and histidine residues residing in the heavy chain of the mature polypeptide.

The invention therefore includes a polypeptide having similarity with the amino acid sequence of SEQ ID NO: 1. The two amino acid sequences (i.e., the amino acid sequence of the polypeptide and the sequence of SEQ ID NO: 1) are aligned such that the residues are aligned to maximize the number of amino acids that they have in common along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to maximize the number of shared amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. The percentage amino acid identity is the higher of the following two numbers: (a) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in SEQ ID NO: 1, multiplied by 100; or (b) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in the candidate polypeptide, multiplied by 100.

A polypeptide of the invention also can include an active analog or active fragment thereof of a polypeptide containing amino acid 1 to amino acid 843 of

SEQ ID NO: 1. An active analog or active fragment preferably is characterized by the same amidolytic activity properties with respect to a target polypeptide as is the polypeptide shown in SEQ ID NO: 1.

An active analog of the invention includes a polypeptide having one or
5 more amino acid substitutions that do not eliminate amidolytic activity for a target polypeptide. For example, an active analog of the invention is characterized by the ability to cleave any denatured polypeptide and nondenatured serpin polypeptide in the RSL region of the serpin polypeptide.

Substitutes for an amino acid in the polypeptides of the invention may be
10 selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include
15 arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of preferred conservative substitutions include Lys for Arg and *vice versa* to maintain a positive charge; Glu for Asp and *vice versa* to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH₂.

20 Analogs, as used herein, also include modifications. Modified cysteine proteinases include a cysteine proteinase that is chemically and enzymatically derivatized at one or more constituent amino acid, including side chain modifications, backbone modifications, and N and C- terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment
25 of carbohydrate or lipid moieties, cofactors, and the like. Modified cysteine proteinases of the invention will retain amidolytic activity with regards to a target polypeptide. For example, a modified cysteine proteinase of the invention will hydrolyze the peptide bond in the RSL region of a human α_1 -PI polypeptide at glutamic acid and alanine, and the phenylalanine and leucine
30 peptide bond as described above and as shown in SEQ ID NO: 1.

Fragments of a cysteine proteinase of the invention include a portion of a cysteine proteinase containing deletions or additions of one or more contiguous

or noncontiguous amino acids such that the resulting polypeptide still retains amidolytic activity with respect to a target polypeptide. An example of an active fragment of a polypeptide of the invention is the catalytic domain of the heavy chain polypeptide containing amino acid 148 to amino acid 629 SEQ ID NO: 1.

5 The polypeptide of the invention can be obtained by several methods. Isolation of the polypeptide present on the surface of a cell producing the proteinase typically requires lysis of the cell followed by purification. Such purification methods are well known in the art and the following represent nonlimiting examples of suitable protein purification procedures: fractionation
10 on immunoaffinity, ion-exchange, hydroxyapatite, Phenyl-Sepharose HP, MonoQ HR 5/5, or MonoP columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an ion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75. Preferably, isolation of the polypeptide
15 from *P. gingivalis* is accomplished using the parameters set forth in Table 1 (acetone precipitation, Sephadex G-150, Mono-Q FPLC, and TSK gel filtration) or a combination of hydroxyapatite, Phenyl-Sepharose HP, MonoQ HR 5/5 and MonoP column chromatography steps as described herein.

A cysteine proteinase of the invention may also be isolated from
20 organisms other than *P. gingivalis*. Other organisms can express a cysteine proteinase that is encoded by a coding region having similarity to the mature polypeptide coding region. A "coding region" is a linear form of nucleotides that encodes a polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of a coding region are
25 generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. "Regulatory region" refers to a nucleic acid fragment that regulates expression of a coding region to which a regulatory region is operably linked. Nonlimiting examples of regulatory regions include promoters, transcription initiation sites, translation start sites, translation stop sites, and
30 terminators. "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulatory element is "operably linked" to a coding region when it is

joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory region.

Alternatively, other organisms can express a cysteine proteinase from a recombinant coding region encoding the proteinase. The identification of similar coding regions in other organisms can be accomplished as described herein. A cysteine proteinase can be isolated using purification methods that are well known in the art. Alternatively, the proteinase can be chemically synthesized using methods that are well known in the art including, for instance, solid phase synthesis. Examples of, for instance, coding and regulatory regions are described herein.

The expression of periodontain from *P. gingivalis* and subsequent cleavage of a specific substrate results in a free amino acid or a free leaving group, each of which can be assayed using techniques known to those of skill in the art. Other methods can be based on immunogenic properties of periodontain, for instance immunoassays and histochemistry, the detection of mRNA, and PCR related methods, all of which are known to one of skill in the art.

As described in the Examples, the amino acid sequence of the amino-terminal end of a heavy chain polypeptide was used to identify the nucleotide sequence of the periodontain coding region (SEQ ID NO: 2). The nucleotide sequence was present in a publically available database containing the nucleotide sequence of the partially finished *P. gingivalis* W83 genome. The nucleotide sequence of the genome of this bacterium is currently being determined by the Institute for Genomic Research and is available at www.tigr.org. However, even though the nucleotides that encode the *P. gingivalis* cysteine proteinase are known, there has been no indication that the nucleotides were in fact transcribed and translated. The data obtained from the database only contained the nucleotide sequence of a genomic clone; there was no disclosure that the nucleotides did or did not contain an open reading frame. Moreover, there is little data known to the art regarding regulatory regions required for either the transcription or the translation of a nucleotide sequence in *P. gingivalis*.

Thus, a person of ordinary skill, having the nucleotide sequence of the genomic clone, would not be able to predict that the open reading frame encoding periodontain was transcribed or translated. Moreover, even if there was a suggestion that the open reading frame was both transcribed and translated, there is no suggestion that the polypeptide encoded by the open reading frame would have the novel activity of periodontain.

Accordingly, the present invention is directed to a nucleic acid fragment encoding a polypeptide, particularly a cysteine proteinase, active analog (including active modification) or active fragment thereof. The nucleic acid fragment can have a nucleotide sequence as shown in SEQ ID NO: 2. Alternatively, nucleic acid fragments of the invention include those whose complement hybridize to SEQ ID NO: 2 under standard hybridization conditions as described herein. During hybridization the entire nucleotide sequence of the complement can hybridize with SEQ ID NO: 2. Preferably, at least about 15 nucleotides of the complement hybridize with SEQ ID NO: 2, more preferably at least about 50 nucleotides, most preferably at least about 100 nucleotides.

Alternatively, the nucleic acid fragment can have a nucleotide sequence encoding a polypeptide having the amino acid sequence or a fragment thereof as shown in SEQ ID NO: 1. An example of the class of nucleotide sequences encoding such a polypeptide is SEQ ID NO: 2. This class of nucleotide sequences is large but finite, and the nucleotide sequence of each member of the class can be readily determined by one skilled in the art by reference to the standard genetic code.

The identification of similar coding regions in other organisms can be accomplished by screening individual wild-type microorganisms for the presence of nucleotide sequences that are similar to the coding region of periodontain, which is shown in SEQ ID NO: 2. Screening methods include, for instance, hybridization of a detectably labeled probe with a nucleic acid fragment.

Standard hybridizing conditions are a modification of the conditions used by Church et al., Proc. Natl. Acad. Sci., (USA) 81:1991 (1984): 0.5 M phosphate buffer, pH 7.2, 7 % SDS, 10 mM EDTA, at 68° C. and three washes, each for 20

minutes in 2x SSC, 0.1 % SDS, at 65° C. Preferably, a primer will hybridize to the nucleotide sequence set forth in SEQ ID NO: 2 under standard hybridizing conditions. Generally the primer does not have to be complementary to all the nucleotides of the nucleic acid fragment as long as there is hybridization under the above-stated conditions.

“Complement” and “complementary” refer to the ability of two single stranded nucleic acid fragments to base pair with each other, where an adenine on one nucleic acid fragment will base pair to a thymine on a second nucleic acid fragment and a cytosine on one nucleic acid fragment will base pair to a guanine on a second nucleic acid fragment. Two nucleic acid fragments are complementary to each other when a nucleotide sequence in one nucleic acid fragment can base pair with a nucleotide sequence in a second nucleic acid fragment. For instance, 5'-ATGC and 5'-GCAT are complementary. The term complement and complementary also encompasses two nucleic acid fragments where one nucleic acid fragment contains at least one nucleotide that will not base pair to at least one nucleotide present on a second nucleic acid fragment. For instance, the third nucleotide of each of the two nucleic acid fragments 5'-ATTGC and 5'-GCTAT will not base pair, but these two nucleic acid fragments are complementary as defined herein. Typically two nucleic acid fragments are complementary if they hybridize under the standard conditions referred to herein.

Preferred oligonucleotide primers are nucleic acid fragments complementary to a coding region or another nucleotide sequence that encodes an isolated cysteine peptidase. For instance, a primer can contain a consecutive series of nucleotides complementary to a portion of the nucleotide sequence encoding the polypeptide or a fragment thereof of SEQ ID NO: 1. Preferably, an oligonucleotide primer is about 15 base pairs, more preferably about 17 base pairs in length. Preferred oligonucleotide primers are degenerative primers. Such degenerative primers can be prepared in accordance with any region of interest as set forth in SEQ ID NO: 2. Methods of detectably labeling an oligonucleotide primer are well known to the art.

The nucleic acid fragment that is identified by the prepared primer is further analyzed to determine if it encodes a polypeptide with amidolytic activity for a target polypeptide as defined herein. Another method for screening individual microorganisms for the presence of nucleotide sequences that are similar to the coding regions of the present invention is the polymerase chain reaction (PCR).

Individual wild-type microorganisms containing nucleic acid fragments encoding a polypeptide of the invention can also be identified using an antibody. Preferably the antibody is directed to the cysteine proteinase periodontain. The production of antibodies to a particular polypeptide is known to a person of skill in the art, and is further detailed herein.

The use of hybridization of an oligonucleotide primer to a coding region present in individual wild-type microorganisms can be used as a method to identify the nucleotides of the coding region identical or similar to a coding region present in SEQ ID NO: 2. Two nucleic acid sequences are "similar" if the two nucleic acid sequences can be aligned so that a percentage of corresponding residues are identical. The two nucleotide sequences (i.e., the nucleotide sequence of the coding region and the nucleotide sequence of the coding region of SEQ ID NO: 2) are aligned such that the nucleotides of the start and stop codons are in register, then further aligned to maximize the number of nucleotides that they have in common along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to place the nucleotides of the start and stop codons in register and to maximize the number of shared nucleotides, although the nucleotides in each sequence must nonetheless remain in their proper order. The percentage nucleotide identity is the higher of the following two numbers: (a) the number of nucleotides that the two sequences have in common within the alignment, divided by the number of nucleotides in SEQ ID NO: 2, multiplied by 100; or (b) the number of nucleotides that the two sequences have in common within the alignment, divided by the number of nucleotides in the candidate coding region, multiplied by 100. Preferably, two nucleic acid sequences have at least about 50%, more preferably at least about 70%, and most preferably at least about 90% identity.

The coding region can then be isolated and ligated into a vector as described below.

As mentioned above, a nucleic acid fragment of the invention can be inserted in a vector. Construction of vectors containing a nucleic acid fragment of the invention employs standard ligation techniques known in the art. See, e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989) or Ausubel, R.M., ed. *Current Protocols in Molecular Biology* (1994). A vector can provide for further cloning (amplification of the nucleic acid fragment), i.e., a cloning vector, or for expression of the polypeptide encoded by the coding region, i.e., an expression vector. The term vector includes, but is not limited to, plasmid vectors, viral vectors, cosmid vectors, or artificial chromosome vectors. Typically, a vector is capable of replication in a bacterial host, for instance *E. coli*. Preferably the vector is a plasmid.

Selection of a vector depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, vector replication rate, and the like. Suitable plasmids for expression in *E. coli*, for example, include pUC(X), pKK223-3, pKK233-2, pTrc99A, and pET-(X) wherein (X) denotes a vector family in which numerous constructs are available. pUC(X) vectors can be obtained from Pharmacia Biotech (Piscataway, NH) or Sigma Chemical Co. (St. Louis, MO). pKK223-3, pKK233-2 and pTrc99A can be obtained from Pharmacia Biotech. pET-(X) vectors can be obtained from Promega (Madison, WI), Stratagene (La Jolla, CA), and Novagen (Madison, WI). To facilitate replication inside a host cell, the vector preferably includes an origin of replication (known as an "ori") or replicon. For example, ColE1 and P15A replicons are commonly used in plasmids that are to be propagated in *E. coli*.

An expression vector optionally includes regulatory regions (e.g., promoters) operably linked to the coding region. The invention is not limited by the use of any particular promoter, and a wide variety are known. Promoters act as regulatory signals that bind RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding region. The promoter used in the invention can be a constitutive or an inducible promoter. It can be, but need not

be, heterologous with respect to the host cell. Preferred promoters for bacterial transformation include *lac*, *lacUV5*, *tac*, *trc*, T7, SP6 and *ara*.

An expression vector can optionally include a Shine Dalgarno site (e.g., a ribosome binding site), and a start site (e.g., the codon ATG) to initiate translation of the transcribed message to produce the enzyme. It can also include a termination sequence to end translation. A termination sequence is typically a codon for which there exists no corresponding aminoacyl-tRNA, thus ending polypeptide synthesis. The nucleic acid fragment used to transform the host cell can optionally further include a transcription termination sequence.

10 The *rrnB* terminators, which is a stretch of DNA that contains two terminators, T1 and T2, is an often used terminator that is incorporated into bacterial expression systems (J. Brosius et al., *J. Mol. Biol.*, 148:107-127 (1981)).

The nucleic acid fragment used to transform the host cell optionally includes one or more marker sequences, which typically encode a polypeptide that inactivates or otherwise detects or is detected by a compound in the growth medium. For example, the inclusion of a marker sequence can render the transformed cell resistant to an antibiotic, or it can confer compound-specific metabolism on the transformed cell. Examples of a marker sequence are sequences that confer resistance to kanamycin, ampicillin, chloramphenicol, and tetracycline.

20

Antibodies can be produced to a polypeptide having the sequence of SEQ ID NO: 1 or a polypeptide having a percentage amino acid identity of SEQ ID NO: 1 as described herein. Alternatively, antibodies can be made to an antigenic analog or antigenic fragment of a polypeptide having the sequence or a fragment sequence thereof of SEQ ID NO: 1. An antigenic analog (including antigenic modifications), and antigenic fragment of a polypeptide derived from SEQ ID NO: 1 is one that generates an immune response in an animal (i.e., it does not eliminate peptide antigenicity in an animal). Analogs and fragments used in this context are as defined above with respect to active analogs and active fragments.

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Accordingly, an aspect of the invention is an immunogenic composition comprising an isolated cysteine proteinase, or an antigenic analog or antigenic fragment thereof, preferably the cysteine proteinase periodontain. The cysteine

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proteinase of the invention preferably has amidolytic activity to cleave, i.e., hydrolyze, a peptide bond in a target polypeptide.

The immunogenic composition can further include excipients or diluents that are pharmaceutically acceptable as carriers and compatible with the immunogenic composition. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other ingredients of a composition and not deleterious to the recipient thereof. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the immunogenic composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the immune-stimulating composition.

The immunogenic composition can be used in a method for protecting an animal from a disease caused by *P. gingivalis*. This method includes administering the immunogenic composition and eliciting antibodies to a cysteine proteinase, antigenic analog, antigenic fragment, or antigenic modification. The diseases that can be treated in this manner include periodontal diseases, which includes gingivitis and periodontitis. Clinical hallmarks of periodontitis include lose of tooth attachment and periodontal pocket formation.

Alternatively and preferably, periodontal diseases can be treated by the use of inhibitors of a cysteine proteinase. An inhibitor of a cysteine proteinase, preferably periodontain, can be present in a composition that preferably contains a pharmaceutically acceptable carrier. For instance, inhibitors can be applied systemically, subgingivally (e.g., subgingival irrigation) and/or by controlled release delivery directly into the periodontal pocket using methods well known to the art (see, e.g., Kornman, K., *J. Periodontol.*, 64:782-791 (1993)). Preferably, an inhibitor is applied subgingivally or by controlled release delivery.

The cysteine proteinase, active analogs and active fragments thereof can be used in a method of reducing growth of bacteria *in vitro* or *in vivo*. Preferably, the bacteria is a periodontal pathogen, i.e. a bacterial pathogen that

causes periodontal disease, more preferably the bacteria is *P. gingivalis*. The inability of asaccharolytic *P. gingivalis* to utilize free amino acids makes the bacterium entirely dependant on an external peptide supply. The action of the polypeptides of the invention may be required for bacterial growth, and
5 inhibition of the polypeptides of the invention may inhibit the *in vivo* growth of organisms, including *P. gingivalis*.

The present invention also includes a method for identifying and isolating an inhibitor of a polypeptide of the invention. The method includes identifying an agent that inhibits the amidolytic activity of the polypeptide by
10 incubating the polypeptide with the agent under conditions that promote amidolytic activity of the polypeptide and determining if the amidolytic activity of the polypeptide is reduced relative to the amidolytic activity of the polypeptide in the absence of the agent.

15

Examples

Objects and advantages of this invention are further illustrated by the following examples, but the particular materials and amounts thereof recited in these examples, as well as other conditions and details, should not be construed to unduly limit this invention.

20

Example 1

25 **Isolation and characterization of a proteinase derived from *P. gingivalis***

Materials and Methods

Reagents

Diisopropyl fluorophosphate, leupeptin, and 3,4 dichloroisocoumarin
30 were purchased from Calbiochem, La Jolla, CA. All other reagents, unless otherwise indicated, were obtained from Sigma Chemical Company, St. Louis, MO. All reagents were of at least analytical grade.

Bacteria Cultivation

The *P. gingivalis* (HG66) strain (Dr. Roland Arnold, University of North Carolina, Chapel Hill, NC) was used for the purification of periodontain. Cells were grown anaerobically in 5 liters (L) of broth containing 150 grams (g) of trypticase soy broth, 25 g yeast extract (both from Difco, Detroit, MI), 25 milligrams (mg) hemin, 2.5 g cysteine, 0.5 g dithiothreitol (DTT), and 5 mg of menadione (all from Sigma), at 37 °C for 24 hours (h) in an atmosphere of 85% N₂, 10% CO₂, and 5% H₂. The seed culture was used to inoculate 100 L of the same broth and the bacterium was then grown in a 130 L fermentor (W.B. Moore, Inc., Easton, PA) at the University of Georgia Fermentation Plant. Cells were grown for 24 hours until late stationary phase of bacterial growth (OD₆₆₀ > 2.0). Additional strains, 2561 (ATCC Accession No: 33277), W50 (ATCC Accession No: 53978) (both from ATCC, Bethesda, MD), W12, and 381 (both from Dr. Caroline A. Genco, Boston University Medical School, Boston MA), were grown under the conditions described above in 1 L volumes.

Cellular Localization

Whole cell culture mixtures were fractionated to determine localization of periodontain. Initially, a low speed centrifugation (6,000 x g, for 20 minutes at 4 °C) was used to pellet the cells, after which the supernatant was subjected to high speed ultracentrifugation (100,000 x g, for 120 minutes at 4 °C) to separate vesicles from the supernatant which contained all soluble proteins. For quantitation of proteolytic activity all samples were brought to an equal volume by the addition of 50 millimolar (mM) Tris, pH 7.4.

α₁-PI Inactivation Assays

Detection of the proteolytic activity of periodontain was determined by following periodontain's ability to specifically inactivate α₁-PI. using α-chymotrypsin as a target proteinase as described by Nelson et al., Anal. Biochem., 260:230-236 (1998). Briefly, native α₁-PI (0.15 nanomoles (nmols)) was mixed with samples containing a putative inactivating activity in assay

buffer (50 mM Tris, 10 mM cysteine, pH 7.8 and allowed to incubate at time intervals of 0, 30, 60, 90, 120, 240 minutes after which an equimolar amount of α -chymotrypsin (0.15 nmols) was added to complex any remaining functional α_1 -PI. The chymotrypsin substrate, N-Suc-Ala-Ala-Pro-Phe- *para*-nitroanalide in dimethyl sulfoxide, was added and, after a 4 minute incubation at 25°C, the reaction was stopped by the addition of 50 μ l glacial acetic acid. Endpoint absorbance at 405 nm was read in a Molecular Devices SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA) and the percentage of α_1 -PI remaining was calculated from controls (no α_1 -PI, 0.15 nM α -chymotrypsin) versus (0.15 nM α_1 -PI, 0.15 nM α -chymotrypsin) in the 4 minute reaction with N-Suc-Ala-Ala-Pro-Phe- *para*-nitroanalide.

Inactivation of α_1 -PI by periodontain was further determined by electrophoresis. Briefly, α_1 -PI (2 μ M) was incubated with periodontain (2 nM) for 0, 15, 30, 45, and 60 minutes before the reaction was stopped by the addition of an SDS sample buffer. A control sample was preincubated with 100 μ M of L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64) for 5 minutes prior to 60 minutes incubation with α_1 -PI. The samples were electrophoresed on a 12% separating gel. Subsequently, samples were separated on a 16% peptide gel to isolate a 3 kD fragment produced by this cleavage. Referring to SEQ ID NO: 4 and Figures 4 and 6, both N-terminal amino acid sequence analysis and mass spectroscopy of the isolated fragment revealed that the major cleavage site was after the glutamic acid residue and a minor cleavage site was after the phenylalanine relative to the Methionine-Serine bond which forms the "bait" region of the reactive site loop (RSL) and is attacked by HNE at the (*) position.

Proteinase purification

Bacteria were harvested at stationary phase, and the cells were removed by continuous centrifugation (Sharples AS-16P rotor, Alfa Laval Separation Inc., Warminster, PA). The cell-free culture fluid was concentrated to 5 L in a Pellicon system (Millipore, Bedford, MA) using a 30 kD molecular weight (MW) cut-off membrane, and then precipitated with 7.5 L of acetone at -10 °C.

The protein pellet was redissolved in 20 mM Bis-Tris, 150 mM NaCl, 0.02% NaN₃, pH 6.8 (buffer A), supplemented with 1.5 mM 4,4'-dithiopyridine disulfide, and dialyzed overnight against the same buffer in a 10 kD MW cut-off membrane (Spectra-Por, Spectrum Corp., Laguna Hills, CA) with two additional changes of buffer A supplemented with 5 mM CaCl₂. The dialyzed fraction was clarified by centrifugation (40,000 x g, for 30 minutes), concentrated by ultrafiltration (Amicon PM-10 membrane, Millipore, Bedford, MA) and applied in 20 ml fractions, each representing 5 L of starting supernatant, to a Sephadex G-150 column (5 x 105 centimeters (cm)) (Amersham-Pharmacia, Uppsala, Sweden) equilibrated with buffer A, at a flow rate of 30 milliliter/hour (ml/h). The activity was pooled, dialyzed against 50 mM Tris, pH 7.4 (buffer B), and further purified by ion exchange chromatography on a Mono-Q column (FPLC system, Amersham-Pharmacia, Uppsala, Sweden), with elution in a linear gradient of 0 mM to 500 mM NaCl in buffer B. Activity was concentrated and final purification obtained by separation on a TSK-GEL G3000SW (TosoHaas, Montgomeryville, PA) column using 50 mM Tris, 200 mM NaCl, pH 7.4.

20 Electrophoresis

Enzyme purification and visualization of the heavy and light chains were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% separating gel using the Tris-HCl/Tricine buffer system, according to Schagger et al., *Anal. Biochem.*, **166**:368-379 (1987)). A nondenaturing PAGE (Davis, *Ann. N.Y. Acad. Sci.*, **121**:404-427 (1964)) in a 4-20% gradient gel was used to show the native protein as a single band.

Molecular Mass Determination

The mass of the native enzyme was determined by gel filtration using a TSK-GEL G3000SW (TosoHaas, Montgomeryville, PA) calibrated with gel filtration standards (Bio-Rad, Richmond, CA). The mass of the separated heavy and light chains from SDS-PAGE were estimated by scanning the gel using the

Eagle Eye II imaging system (Stratagene, La Jolla, CA) and calculating a linear regression of low molecular weight electrophoresis standards (Amersham-Pharmacia, Uppsala, Sweden) as reference. Accurate molecular mass measurements of the digestion products of α_1 -PI after enzymatic inactivation employed the use of matrix assisted laser desorption ionization (MALDI), with mass spectra acquired using a Vestec MALDI Linear Time-of-Flight Mass Spectrometer (Perspective Biosystems, Perkin-Elmer, Foster City, CA) at the Mass Spectroscopy Facility (University of Georgia, Athens, GA) according to the manufacturer's instructions.

10

Protein Sequence Analysis

For amino-terminal sequence analysis, proteins resolved by electrophoresis were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane according to Matsudaira (Matsudaira, *J. Biol. Chem.*, **262**(21):10035-10038 (1987)). Sequence analysis was performed with an Applied Biosystems 4760A gas-phase sequencer at the Molecular Genetics Instrumentation Facility (University of Georgia, Athens, GA) operated according to the manufacturer's recommendations.

20 Inhibition studies

For inhibition studies, periodontain was used at a concentration which was capable of completely inactivating 0.15 nmols of α_1 -PI in our standardized assay (Nelson et al., *Anal. Biochem.*, **260**:230-236 (1998)) after one hour of incubation. Representatives of the various classes of proteinase inhibitors, at indicated concentrations, were preincubated with enzyme for 5 minutes prior to the addition of α_1 -PI. The cleavage of inhibitor (%) was normalized to a native inhibitor control in order to give the relative percent inactivation for each compound or protein tested against periodontain. The compound, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), which stoichiometrically inhibits cysteine proteinases of the papain family, was used to titrate periodontain. This allowed us to quantitate stock solutions of

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periodontain in terms of active enzyme, and make dilutions to desired concentrations for the degradation experiments described below.

Protein and peptide degradation by periodontain

5 The degradation of proteins was followed by using either native lysozyme or reduced-carboxymethylated-maleylated lysozyme (lysozyme-RCM), (30 micromolar (μ M)). Either protein was incubated with periodontain (30 nM) in a final volume of 20 microliter (μ l) of assay buffer for 0 and 15 minutes, 1 hour and 24 hours. The reaction was stopped by addition of 20 μ l of
10 SDS sample buffer (4% SDS, 20% glycerol, 0.125 M Tris-HCl, pH 6.8), followed by boiling for 5 minutes, after which the entire sample was electrophoresed on a 12% gel and stained in 0.1% Coomassie Brilliant Blue to visualize the protein bands.

 To determine the cleavage sites within the reactive site loop (RSL) of α_1 -
15 PI, inhibitor (20 μ g) was incubated with 1 μ g of periodontain for 4 hours, after which the sample was subjected to 16% SDS-PAGE to separate the approximately 3 kD fragment obtained by cleavage within the loop. The fragment was analyzed for both amino terminal sequence and molecular mass using an automated protein sequencer and mass spectroscopy, respectively, as
20 described above.

 For analysis of the fragments obtained through digestion of the insulin β -chain, periodontain (8 nM) was incubated with this peptide substrate (40 μ M) in assay buffer in a final volume of 90 μ l for desired time intervals. After stopping the reaction by the addition of 10 μ l of 10 normal (N) HCL, samples were
25 centrifuged (10,000 x g, 2 minutes) and the entire supernatant (100 μ l) was subjected to reverse-phase HPLC. Sample application to a Beckman Ultrasphere 5 μ m ODS column (4.6 x 250 mm) (Beckman-Coulter Instruments, Fullerton, CA) equipped with an Ultrasphere 5 μ m ODS guard-column (4.6 x 45 mm) (Beckman-Coulter Instruments, Fullerton, CA) was carried out in 0.1%
30 TFA in water (solvent A) and separations were performed with a linear gradient

of 0.08% TFA in 80% acetonitrile/water (solvent B) over 40 minutes at a flow rate of 1 ml/minute. The peptide elution was monitored at 215 nm.

Gelatin Zymograph

- 5 Zymography analysis on gelatin gels was performed on pure samples of periodontain in the presence of 5 mM cysteine, with or without 100 μ M of E-64 (samples were incubated with E-64 for about 5 minutes). After the addition of SDS sample buffer, the samples were subjected to electrophoreses at 4 °C on a 10% SDS-PAGE with gelatin (Difco, Detroit MI) (0.1 mg/ml) incorporated into
- 10 the gel. Following electrophoresis, the gel was washed twice with 2.5% weight/volume (w/v) Triton X-100 to remove the SDS and then incubated in activation buffer (50 mM Tris, 20 mM cysteine, pH 7.4) at 37 °C for two hours. The zymograph was developed in 0.1% Amido Black, with clearing zones (negative staining) indicating proteolytic digestion of the incorporated gelatin.
- 15 The gelatin zymograph indicated that the periodontain heavy chain (55 kD) contained the catalytic active site.

Cloning of gene fragment encoding N-terminus of periodontain

- Based on the N-terminal sequence of the heavy chain (55 kD) of
- 20 periodontain (SEQ ID NO: 1), a pair of degenerate oligonucleotide primers (5'-ACNGA(G/A)GGNGTNCCNGC-3') (SEQ ID NO: 7) and (5'-NCGCATNGG(G/A)TC(G/A)TT-3') (SEQ ID NO: 8) corresponding to amino acid residues (TEGPA) (SEQ ID NO: 9) and amino acid residues (NDPMR) (SEQ ID NO: 10), respectively, were designed. The DNA fragment coding for
- 25 the N-terminus of periodontain was amplified by PCR using *Pwo* DNA Polymerase (Boehringer-Mannheim/Hoffman LaRoche Ltd., Basel, Switzerland). and 10 nanograms (ng) of W50 *P. gingivalis* DNA (purified by the Purgene kit, Gentra Systems Inc., Plymouth, MN). PCR was carried out with 500 ng of primers for 1 minute at 94 °C, 1 minute at 65 °C, and 20 seconds at 72
- 30 °C. The expected product of 69 basepairs was purified from a 2% agarose gel with the Ultrafree MC Millipore Filter (Millipore, Bedford, MA), phosphorylated at the 5' end with polynucleotide kinase (PNK), and blunt end

ligated into a *SmaI* digested pUC19 vector. The fragment coding for the N-terminus of periodontain was identified by sequence analysis.

Identification of the periodontain gene

5 A database containing the unfinished *P. gingivalis* W83 genome (available from the National Center for Biotechnology Information (NCBI), Unfinished Microbial Genomes at www.ncbi.nlm.gov was searched for the presence of nucleotide sequences corresponding to the NH₂-terminal amino acid sequences of both chains of periodontain using the TBALSTN algorithm
10 (Altschul et al., *Nucleic Acid Res.*, **25**:3389-3402 (1997)). The sequence of the clone: gnl | TIGR | *P. gingivalis*_112, which contained the translated sequence for both chains of periodontain, was retrieved from The Institute for Genomic Research (TIGR) database (<http://www.tigr.org>). The gene encoding
15 periodontain was identified using the NCBI open reading frame (ORF) finder program (also found at NCBI) and the amino acid sequence, obtained by conceptual translation of the entire ORF, was further used for homology screening performed with the NCBI BLAST search tool.

20 **Results**

Enzyme Purification

Previous experience in purifying enzymes from culture supernatants of *P. gingivalis* indicated that ice cold acetone precipitation as an initial step was successful in separating active proteinases from the bulk of peptides and proteins
25 present in or released into the growth medium. Similarly, G-150 gel filtration as an early step was also utilized (Chen et al., *J. Biol. Chem.*, **267**(26):18896-18901 (1992); Pike et al., *J. Biol. Chem.*, **269**(1):406-411 (1994)), both to resolve proteins into rough molecular weight fractions and to remove the excess heme and phytochrome that coprecipitate during acetone treatment. Subsequent anion
30 exchange chromatography (Mono Q) and gel filtration (TSK) allowed periodontain to be purified to near homogeneity. Our yield of 11%

corresponded to over 1 mg of pure protein per each 5 L of starting culture fluid (Table I).

Table I.
Purification of *P. gingivalis* Periodontain

	Fraction Step	Volume	Total	Total	Specific	Purific	Yield
			Activity	Protein	Activity	a-tion	
		<i>ml</i>	<i>Units^a</i>	<i>mg</i>	<i>units/mg</i>	<i>fold</i>	<i>%</i>
	Culture Fluid	15,000	11,000	~300,000	.04	1.00	100
	Acetone Precipitation	60	8,100	3,500	2.3	58	74
	Sephadex G- 150	120	6,550	32.3	203	5,070	60
	Mono-Q, FPLC	5	1,958	5.40	363	9,065	18
	Gel Filtration, TSK	6	1,200	3.26	368	9,203	11

^aBased on 1 unit = the activity necessary to completely inactivate 0.15 nmol of α_1 -PI in 60 minutes at 37°C using the assay described above.

Physical properties

TSK gel filtration of the pure enzyme yielded a single protein peak that eluted with a MW of approximately 75 kD, based on a linear regression data analysis from standards (data not shown). However, a 10% SDS-PAGE containing 1 µg of pure periodontain yielded two distinct bands at 55 kD (heavy chain) and 20 kD (light chain), suggesting periodontain, as a native protein, is a heterodimer (data not shown). Testing a nondenaturing PAGE containing 1 µg pure periodontain and 5 µg of albumin, albumin formed dimers, and to a lesser extent, trimers when subjected to electrophoresis. Conversely, periodontain, under nondenaturing conditions, did not yield any bands indicating a dimer or trimer (data not shown).

Amino terminal sequence analysis of each of these subunits yielded the sequences of TEGVPAEVHALMDNGHFANDPMR (SEQ ID NO: 11) and DEWKKIGSVSVK (SEQ ID NO: 12) for the heavy (55 kD) and light (20 kD) chains, respectively. Analysis of the single protein species isolated from the nondenaturing PAGE, gave two new amino terminal sequences in equimolar quantities, which corresponded to those described above, confirming that periodontain is a heterodimer. It should also be noted that either heating of the sample and/or presence of SDS in the gel buffer was sufficient to separate the heavy and light chains on electrophoresis, suggesting that the native heterodimer was stabilized by ionic interactions rather than a disulfide bridge or covalent bonds. Isoelectric focusing yielded a pI of 5.3 for the native protein (data not shown) while gelatin zymography indicated that the 55 kD heavy chain contained the catalytic active site but the 20 kD light chain was devoid of enzymatic activity.

Stability

Periodontain activity was detected over a broad pH range of about 6.0 to about 9.0, with the optimum being between a pH of about 7.5 to about 8.0. The enzyme was stable at 37 °C, overnight, and at 4 °C for several weeks, when stored in the absence of cysteine. The presence of reducing agent resulted in a
5 50% loss of activity at 37 °C overnight, presumably because of autodigestion. Heating to 60 °C caused complete loss of activity. Samples were routinely stored at -80 °C for several months with less than a 10% loss in activity.

Activation and inhibition

10 Periodontain was completely inactive in the absence of reducing agents, whereas full activity was achieved with either free cysteine, β -mercaptoethanol, dithiothreitol (DTT), or dithioerythritol (DTE) at 0.1 mM concentration. Unlike the gingipains, which have higher activity in free cysteine, no single reducing agent was superior in activating periodontain. Furthermore, increasing
15 concentrations of these reagents (up to 10 mM) did not cause any additional stimulation of periodontain activity. Finally, calcium ions (Ca^{2+}) did not have a stabilizing effect and glycyl-glycine did not stimulate activity, indicating additional differences between periodontain and the gingipains (data not shown). Based on its requirement for a reducing environment to become active,
20 periodontain can be classified as a cysteine proteinase, and this is confirmed by the fact that it is readily inhibited by common cysteine proteinase inhibitors (Table II). The ability of E-64 to inhibit periodontain suggests that this enzyme is more closely related to members of the papain family than other cysteine proteinases of *P. gingivalis* which are either not inhibited (Kgp) or only weakly
25 inhibited (Rgp's) by this compound.

Table II.
Inhibition Profile of Periodontain

5	Inhibitor	Class	Concentration	Percent Inhibition
	EDTA	Metallo	25 mM	0
	Dichloroisocoumarin	Serine	0.5 mM	2
10	Diisopropylfluorophosphate	Serine	0.5 mM	5
	Leupeptin	Serine/Cysteine	50 μ M	88
15	Tosyl-L-lysine chloromethyl ketone	Serine/Cysteine	5 mM	91
	Phe-Pro-Arg chloromethyl ketone	Serine/Cysteine	10 μ M	96
20	Z-Phe-Lys benzoyloxy methyl ketone	Cysteine	10 μ M	99
	Idoacetamide	Cysteine	5 mM	100
25	E-64	Cysteine	100 μ M	100

Enzyme specificity

30 Periodontain was originally identified as a unique proteinase which was able to inactivate α_1 -PI through proteolytic cleavage. From the 3 kD size difference of cleaved versus native α_1 -PI noted on SDS-PAGE, it was speculated

that periodontain caused hydrolysis within the exposed C-terminal reactive site loop (RSL) of this molecule. Sequencing of the peptide generated by incubation of α_1 -PI with periodontain indicated that cleavage took place after the glutamic acid (amino acid residue 354 of SEQ ID NO: 3), and after phenylalanine (amino acid residue 352 of SEQ ID NO: 3) of the human α_1 -PI. However, screening numerous synthetic pNA substrates with either Glu or Phe specificity in the analogous position yielded no detectable cleavage by periodontain. Indeed, even when the synthetic substrate Phe-Leu-Glu-pNA, which mimics amino acid residues 352, 353 and 354 of the RSL of human α_1 -PI (SEQ ID NO: 3) was employed, no hydrolysis was detected, indicating that a specific amino acid residue at the site of hydrolysis does not dictate the specificity of this enzyme.

Further investigated into the activity of periodontain on protein and peptide substrates was performed to further elucidate cleavage specificity. However, the insulin β -chain was hydrolyzed to such an extent that individual cleavages could not be mapped, even at low E:S molar ratios (1:5000). Rather, the HPLC analysis revealed no less than 10 peptides were generated from this thirty amino acid polypeptide within 15 minute and complete digestion occurred in 60 minutes (data not shown). Next, the activity of periodontain on native proteins was examined. However, the enzyme was unable to degrade azocasein, casein, lysozyme, collagen, fibrin, plasminogen, and fibrinogen (data not shown). In contrast, when lysozyme was reduced, carboxymethylated, and maleylated, complete digestion was noted in less than 10 minutes. These results, together with activity detected on the gelatin zymograph, indicate that periodontain cleaves denatured or easily accessible polypeptide chains, but it cannot cleave whole proteins with defined secondary or tertiary structure, α_1 -PI being the exception.

Structure of the Periodontain Gene

The region encoding the N-terminal sequence of the 55 kD catalytic subunit of periodontain was amplified by PCR using degenerate primers and *P. gingivalis* W50 DNA. The 69 basepair PCR product was cloned and sequenced.

Using this structural information, the gene encoding periodontain was extracted from the unfinished microbial genome at NCBI and found to encode an 843 amino acid residue protein with a calculated molecular weight of 93,127 Da (Figure 1). The predicted size of the translated protein is approximately 20 kD larger than that found experimentally by both gel filtration and SDS-PAGE. However, examination of the gene product revealed that proteolytic processing of the translated protein at Arg-147 and Lys-629 would yield a prepropeptide, a heavy chain containing the active site, and a light chain derived from the C-terminal part of the proprotein that corresponds exactly to the experimentally determined amino terminal sequences for these subunits (Figure 2).

Thus, the native protein would have a predicted molecular weight of 76,727 Da, composed of a 52,981 Da catalytic heavy chain and a 23,764 Da C-terminal light chain, with a calculated pI of 5.18. This is in agreement with the experimental findings of a native protein of about 70 to about 80 kD by gel filtration, composed of a catalytic subunit of 55 kD and a noncatalytic subunit of 23 kD by SDS-PAGE, with a pI of 5.3. These data suggest a potential role in the processing of the pro-form of periodontain by both gingipains R and gingipain K, all of which are abundantly present in *P. gingivalis*.

20 Distribution of periodontain

Using the cloned 69 basepair catalytic amino terminal fragment as a probe, Southern blot analysis on the W50 strain revealed a single hybridizing band with each of the restriction enzyme digests (data not shown). Furthermore, an α_1 -PI inactivating activity was detected in all *P. gingivalis* strains tested. Interestingly, periodontain was most frequently associated with the membrane and outer membrane vesicles in strains 2561, W50, W12, and 381, despite the fact that it was soluble in strain HG66 (Fig. 3). This is also in agreement with the distribution of other *P. gingivalis* proteinases (Chen et al., J. Biol. Chem., 267(26):18896-18901 (1992); Pike et al., J. Biol. Chem., 269(1):406-411 (1994)).

Discussion

The multiple trypsin-like proteolytic activities have a significant contribution to the virulence of *P. gingivalis*, for both invasion and host defense evasion. In support of this concept, deletion of the two genes (*rgpA* and *rgpB*) encoding various forms of gingipains R, has been shown to attenuate *in vitro* virulence of the knockout strain (Nakayama et al., J. Biol. Chem., 270:(40):23619-23626 (1995)). In addition, the use of antibodies to gingipains R polypeptide chain derived fragments has shown *in vivo* protection against *P. gingivalis* infection in a mouse model system (Genco et al., Infect. Immun., 66(9):4108-4114 (1998)).

Despite the importance of these bacterial proteinases, the abundance of host derived metallo-proteinases and serine proteinases, including active HNE, in the gingival crevicular fluid of individuals with severe periodontitis, is still believed to be the primary factor responsible for much of the extracellular matrix destruction which occurs in this disease (Cox et al., J. Periodont. Res., 24:353-361 (1989)). The concentration of HNE within the neutrophil is near 3.0 μM , and it is likely to be as high as 268 μM at sites of inflammation (Travis et al., Am. J. Respir. Crit. Care Med., 150:S143-S146 (1994)), such as those that occur during the development of another major connective tissue disease, pulmonary emphysema.

The regulating inhibitor of HNE is α_1 -PI, a plasma protein which forms a complex with this proteinase and is rapidly removed from the circulation and degraded (Mast et al., J. Biol. Chem., 266(24):15810-15816 (1991)). The inhibitor, however, can itself be inactivated by either oxidation at its reactive site or by proteolytic cleavage by nontarget proteinases within the RSL region (Travis et al., Annu. Rev. Biochem., 52:655-709 (1983)), and it is believed that both mechanisms occur during the development of emphysema. Certainly, the high levels of active HNE in the GCF, despite the presence of α_1 -PI, would suggest that parallel mechanisms for inhibitor inactivation may be also occurring in periodontal disease (Travis et al., Annu. Rev. Biochem., 52:655-709 (1983)). In this respect, it has been reported that whole cells or culture supernatants from

P. gingivalis are capable of proteolytically inactivating α_1 -PI (Grenier, Microbiology, 142:955-961 (1996); Carlsson et al., Infect. Immun., 43(2):644-648 (1984)), although it is clear that this is not due to any of the gingipain -R or -K forms since the inhibitor contains no basic residues within its RSL (Potempa et al., J. Biol. Chem., 274(34):21648-21657 (1998)). Thus, another proteinase(s) must be involved in this process, and it is likely that periodontain serves this purpose.

Periodontain was purified from *P. gingivalis* culture fluids and was found to be a cysteine proteinase that is apparently produced as a heterodimer. This enzyme is similar to the other characterized cysteine proteinases from *P. gingivalis* in that all are primarily secreted in strain HG66, yet present on membranes and vesicles in all other strains. Each has a signal peptide sequence, a long prepropeptide, a large catalytic domain, approximately 50 kD, and an additional C-terminal extension (about 20 kD to about 40 kD). In contrast, whereas the gingipains have a restrictive specificity, periodontain is characterized by an apparently nonspecific proteolysis of peptides or denatured proteins. Although the ability of this enzyme to hydrolyze α_1 -PI, a native protein, is contrary to our premise of its inability to cleave proteins with defined structure, this can be explained by the fact that the RSL is present in a flexible, extended conformation protruding above the protein core (Elliott et al., J. Mol. Biol., 275:419-425 (1998)) and, as such, may mimic a denatured protein or peptide.

From analysis of the N-terminus of the catalytic subunit and subsequent cloning of this fragment, it was possible to elucidate the entire gene sequence from the partially completed genome sequence for *P. gingivalis* obtained from NCBI. Searching the database of known sequences has revealed that periodontain is homologous to both *prtT*, a putative cysteine proteinase from *P. gingivalis*, and streptopain (EC 3.4.22.10), a secreted cysteine proteinase from *Streptococcus pyogenes* (Table III and Figure 1). The *prtT* gene, which has recently been cloned and sequenced (Otogoto et al., Infect. Immun., 61(1):117-123 (1993); Madden et al., Infect. Immun., 63(1):238-247 (1995)), is believed to encode a putative proteinase of a 96 kD to a 99 kD protein. It is not surprising

that periodontain and *priT* have highly homologous sequences, being from the same organism. Indeed, *rgpA* and *rgpB* are two completely separate genes that code for almost identical proteins with significantly overlapping specificity. However, until the gene product for *priT* has been purified and characterized,

5 functional similarities between *priT* and periodontain cannot be assessed.

Table III
Homology of Periodontain with Streptopain and *priT*
(Identity/Similarity)

Domain	Streptopain	<i>priT</i>
Prepropeptide	27/44	35/56
Catalytic Subunit	37/52	52/69
C-terminal Subunit	N/A	24/42
Full Length Protein	N/A	34/52

Periodontain and *priT*, containing 843 and 840 amino acid residues, respectively, are more than twice the size of the streptopain (398 residues) (Figure 1). The function of the light chain of periodontain is presently unknown; however, the C-terminal domain for the putative *priT* gene product has recently

30 been shown to be identical to the putative product for a hemin regulated gene, *hemR* (Karunakaran et al., *J. Bacteriol.*, 179(6):1898-1908 (1997)). Due to the high homology of the catalytic domains of all three genes, we believe that

periodontain most likely should be classified with *prtT* and streptopain as an additional member of the C10 family of cysteine proteinases, as recently outlined in (Barrett, Rawlings, and Woessner, (eds), Handbook of Proteolytic Enzymes, Academic Press, London (1998)).

5 Because *P. gingivalis* is an asacchrolytic organism, it must acquire both carbon and energy predominantly from proteinaceous sources. Recent evidence using radiolabeled substrates has shown that while this organism is very efficient at taking up dipeptides, it is incapable of transporting single amino acids (which may endogenously be present in GCF) across bacterial cell membranes (Dashper
10 et al., J. Dent. Res., 77(5):1133 (1998)). This is supported by the fact that despite a large body of research performed on extracellular endoproteinase from *P. gingivalis*, no aminoproteinase or carboxypoteinase have thus far been described.

 It is thought that the gingipains, which only have a specificity for either
15 Lys-X or Arg-X bonds, would be restricted in their ability to degrade large proteins to the size of dipeptides or tripeptides which could then be transported into the bacterium. Therefore, it is possible that a number of broadly specific proteinases and proteinase, including periodontain, may be physiologically important to *P. gingivalis*, not necessarily as virulence factors, but rather for
20 nutrient acquisition. This may be accomplished by three pathways. First, since periodontain is the only peptidase in *P. gingivalis* so far described which possesses the ability to inactivate α_1 -PI, this maybe a mechanism for increasing the levels of HNE, a nonspecific host proteinase which might be utilized in protein degradation. Second, in combination with HNE, periodontain may
25 augment the degradation of peptides produced by the actions of the gingipains. Third, there is evidence that *P. gingivalis* produces a prolyl dipeptidyl peptidase (Kiyama et al., Bioch. Bioph. Acta, 1396:39-46 (1998)), a prolyl tripeptidyl peptidase, and an uncharacterized collagenase. This proteolytic milieu in the GCF could aid in the final production of peptides capable of being taken up by
30 *P. gingivalis*.

 The complete disclosures of the patents, patent documents, publications, ATCC deposits, and electronically available material (e.g., GenBank amino acid

and nucleotide sequence submissions) etc.. cited herein are incorporated by reference in their entirety as if each were individually incorporated. Various modifications and alterations to this invention will become apparent to those skilled in the art without departing from the scope and spirit of this invention. It should be understood that this invention is not intended to be unduly limited by the illustrative embodiments and examples set forth herein and that such examples and embodiments are presented by way of example only with the scope of the invention intended to be limited only by the claims set forth herein as follows.

WHAT IS CLAIMED IS:

1. An isolated oral bacterial polypeptide which has amidolytic activity for cleavage of a nondenatured human α_1 -proteinase inhibitor at a reactive site loop region of the inhibitor.
2. The isolated polypeptide of claim 1 wherein the polypeptide has amidolytic activity in a solution comprising about 1 mM to about 500 mM Tris, about 500 μ M to about 100 mM cysteine maintained at a pH of about 7 to about 8.
3. The isolated polypeptide of claim 1 which is isolated from *Porphyromonas gingivalis*.
4. The isolated polypeptide of claim 1 which is a cysteine proteinase.
5. The isolated polypeptide of claim 1 which has a molecular weight of about 70 kD to about 80 kD as determined by gel filtration.
6. The isolated polypeptide of claim 1 which cleaves the reactive site loop region of the inhibitor represented by SEQ ID NO: 4 between glutamine and alanine.
7. The isolated polypeptide of claim 6 which cleaves the reactive site loop region of the inhibitor represented by SEQ ID NO: 4 between phenylalanine and leucine.
8. An isolated polypeptide which is an oral bacterial cysteine proteinase and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.

9. The isolated polypeptide of claim 8 wherein the polypeptide has amidolytic activity in a solution comprising about 50 mM Tris, about 20 mM cysteine maintained at a pH of about 7.4 at 37°C.
10. The isolated polypeptide of claim 8 which is isolated from *Porphyromonas gingivalis*.
11. An isolated polypeptide which is isolated from *Porphyromonas gingivalis* and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.
12. The isolated polypeptide of claim 11 wherein the polypeptide has amidolytic activity in a solution comprising about 1 mM to about 500 mM Tris, about 500 μ M to about 100 mM cysteine maintained at a pH of about 7 to about 8.
13. The isolated polypeptide of claim 11 which has a molecular weight of about 70 kD to about 80 kD as determined by gel filtration.
14. The isolated polypeptide of claim 13 comprising an amino acid sequence having a percentage amino acid identity of greater than 37% to that of amino acid 148 to amino acid 843 of SEQ ID NO: 1.
15. The isolated polypeptide of claim 14 comprising an amino acid sequence having a percentage amino acid identity of greater than 52% to that of amino acid 148 to amino acid 629 of SEQ ID NO: 1.
16. The isolated polypeptide of claim 11 which cleaves a target polypeptide nonspecifically.
17. An isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 37% to that of SEQ ID NO: 1.

18. An isolated polypeptide comprising an amino acid sequence represented by SEQ ID NO: 1, an active analog or an active fragment thereof.
19. An isolated polypeptide comprising an amino acid sequence having a percentage amino acid identity of greater than 37% to that amino acid 148 to amino acid 843 of SEQ ID NO: 1
20. An isolated polypeptide comprising an amino acid sequence represented by amino acid 148 to amino acid 843 of SEQ ID NO: 1, an active analog or an active fragment thereof.
21. An isolated nucleic acid fragment encoding an oral bacterial polypeptide which has amidolytic activity for cleavage of a nondenatured human α_1 -proteinase inhibitor at a reactive site loop region of the inhibitor.
22. An isolated nucleic acid fragment encoding a polypeptide which is an oral bacterial cysteine proteinase and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.
23. An isolated nucleic acid fragment encoding a polypeptide which is isolated from *Porphyromonas gingivalis* and has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of the serpin.
24. The isolated nucleic acid fragment of claim 23 wherein the nucleic acid has a nucleotide sequence comprising SEQ ID NO: 2.
25. The isolated nucleic acid fragment of claim 23 wherein the encoded polypeptide comprises an amino acid sequence having a percentage amino acid identity of greater than 37% to amino acid 148 to amino acid 843 of SEQ ID NO: 1.

26. The isolated nucleic acid fragment of claim 23 wherein a complement of the nucleic acid fragment hybridizes to SEQ ID NO: 2 under hybridization conditions of

0.5 M phosphate buffer, pH 7.2, 7% SDS, 10 mM EDTA, at 68° C, followed by three 20 minute washes in 2x SSC, 0.1% SDS, at 65° C, wherein at least about 15 nucleotides of the complement hybridize.

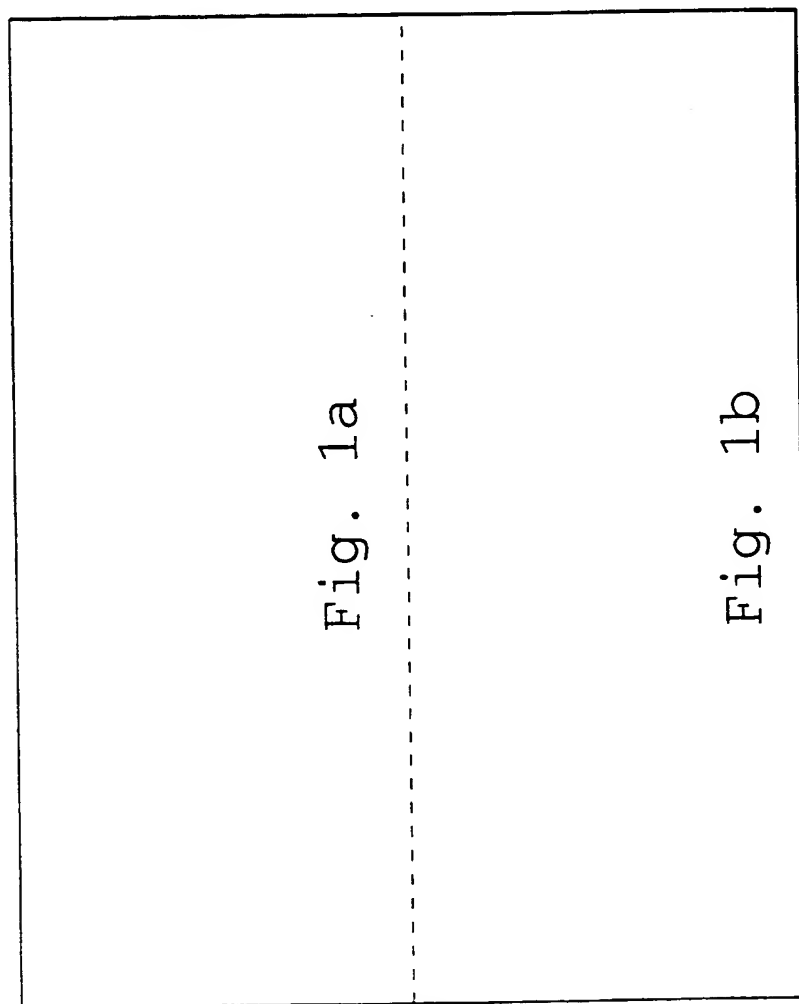
27. A method for identifying an inhibitor of a polypeptide which has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin comprising isolating an agent that inhibits the amidolytic activity of the polypeptide by incubating the polypeptide with the agent under conditions that promote amidolytic activity of the polypeptide and determining if the amidolytic activity of the polypeptide is reduced relative to the amidolytic activity of the polypeptide in the absence of the agent.

28. The method of claim 27 wherein the polypeptide is isolated from *Porphyromonas gingivalis*.

29. An immunogenic composition comprising a polypeptide which has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin that is capable of eliciting antibodies in an animal.

30. A composition comprising an inhibitor to a polypeptide which has amidolytic activity for cleavage of a nondenatured serpin at a reactive site loop region of a serpin isolated from an oral bacterium.

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D NO:

Streptopain	1	MNKKKLCRLSLSLALGGFVLANPVFAQONFARNEKEAKDSATFIQKSAATKAGARSAE
prtt	1	-MKKIFVYTLGLLELLCLPML-QAGPVTSSAEQTAFFAKRQPTL-----SSASIRDD
Periodontain	1	-MKKSELDAEMMLFGHAMQGHAPVTKKHAALSLAK--LALRQVRLRMGQAVVSDKISIDY
Streptopain	61	DIKLDKVNLG-----GELSGNNMYVYNISTG-GFVAVSGDKRSPEILGYSTSGSFDANGKE
prtt	55	VYK--AAER-----EE-----ALEFVENRGEKIGFVLVAADDRFPETGGYAFKGFDAARIP
Periodontain	58	VYRQGDARGITSQEEGSPAIFYVANRGNNEGVALVAADDRIPTLLAYSPIGFEDMDSVP
Streptopain	116	N-----IASFMFSYVEQIKENKALTTVAGTAETQOPVVKSLLDKGIHNNQG
prtt	105	DNLRCMLKGYEREMLAIMDGKAEPIIPLEAKPTRDIPSSIAPIETGEHASDPILWDDQG
Periodontain	118	DNLRMMLQIYEQEIGLTSGKALNEELLE--TEGIPAEVFAHANNHFAHNGHFANDEYRMNQG
Streptopain	164	NPYNLLTPVMEKVKPGEQSFVGOHAATGCVATATAQIMKYHNYPNKGKLDYTYTLSSNNP
prtt	165	YPENTLHPLLP-----SGQAYTGCVATAMQIMRHYKMWPEKISCEYDY YDDMTGT
Periodontain	175	YPMNNKEPLLP-----NGNHAYTGCVATAAQIMRYHSMPLQEGSEEDYHAGSLVG
Streptopain	224	YFNHPKNLFAAISTRQYNNWNNTLPTYSGR-ESNVQKMAHSELMAADVGISVDMMGPPSSGS
prtt	216	HT-----HSGTFGETYNNW SKMPGNISIG-ESPEVKAALSTEMRDVSFSVNMQFAHFGSG
Periodontain	226	N-----WSGTFGEMYDWINMPGNPDIDNHHQSQVDAYATLMRDVSASVSMSSEYENGSG
Streptopain	283	AGSSRVQRALKENFGYNOSSVQINRSDKKQKQMEAQIDKELQNPVYYQGVGVKGHAF
prtt	270	TESISVERALRETFYKLSLYIHRSLPGKEMDMIRKELAENRPVYAGADGSMGHAF
Periodontain	279	TVSVAVVGALRNNFRYKRSLLQLVHVALYTSQEWMDMIRGELASGRPVYAGNNQSMGHAF
Streptopain	343	VIDGADGRNFFHVNWGWGGVSQGFFRLDALNPSEALGTGGCAGGFNGYQSAVVVGIKP----
prtt	330	VCDGYEPDGTTFHFNWGWGGMSNGNFYLNLLNPGSLGTRAGDGGLETDQENVLGIEPASNE
Periodontain	339	VCDGYASDGTTFHFNWGWGGVSNGFALTLTLLSPFSLGIGGEEIGFIYQEEITGTGIEPAKTP

Fig. 1a

prtt 390 VP-GIVPDDPTTLYGQHNMSDEA-LDPSVKIKNYSYAGD~~KLAYRL~~TLPNGTETTNP
 periodontain 399 AEAGTDALPIALKDIEAEYKSESGNAGYSLYNTGEEQSN~~LDLGYRL~~NKADGEVIEVKT

 prtt 448 VVPIVWEDIIIGESTGNIWPCSQFAEGKNTIS~~LYRTDGMADWKEUKHILMGLVNKIEV~~
 periodontain 459 S~~ELINISW~~YG-YGEHPESF~~SAPNQLSQGINTI~~LYRRTGTEQWEPV~~HAQGGYVNSIKV~~

 prtt 508 TMPAGDVAYSVD--~~VPVVKDGSLSHDLKAYS~~SDCK~~ATVYNPGTE~~EFRSRV~~TEALRN~~
 periodontain 518 NTTDPNNVVVTV~~DNNE~~AL~~S~~VPNSFVAD~~LN~~SYEHST~~LVQFN~~SDSP~~EIRHP~~VAFALST

 prtt 565 --TEGRVYFLGRH~~IVE~~HPGDE~~GEKVSL~~ITGLKARAGQYMLVCTGDMESLMEDASWIE~~~~
 periodontain 578 GATADD~~ISLGWV~~AEVPGGSSN-YPVVM~~SKDVL~~TLSEG~~DYTLWYRFS~~IN--NQKDEWKK

 prtt 623 NASTEVAEHTSTHSSLLVASNPQIDLLT~~VHRANPETLP~~ESTTNEGGAT~~FSGK~~IEHVAIK~~~~
 periodontain 635 AGSSVSVKTPTEYTHPLFEVGHNQSTSTY~~EDMAHNRVLP~~DEF~~HLKN~~LG-LPENG~~EVLVFRQ~~

 prtt 683 AFSE~~IF~~-FFQAK~~EEHMS~~AQGETKVLSPE~~TTN~~SSLYTNAELFPDGT~~YIVV~~RR~~EQG-FWDP~~
 periodontain 694 TQSS~~EGSLWAAQET~~TH~~KQGETFVYKPV~~EE-----PIPDGS~~Y~~RA~~TE~~HA~~AFVNGQQQ~~

 prtt 741 ~~V~~DLFCGY~~Y~~RI~~RL~~ITDLSSSDIAGKDVSTIV~~V~~PNPA~~HDVHVAIP~~PTYAGS~~MI~~RL~~LFDIQ~~
 periodontain 745 ~~EY~~LKCKRNY~~T~~V~~NGTAVEAIE--SSEEIR~~EP~~PNPA~~DVVE~~SAPCIPQE~~ASHI~~LFDLS~~

 prtt 801 GRV~~Q~~STKTES~~NDMR~~DVERLPKGT~~YIVV~~DMVGK~~IFIR-~~
 periodontain 803 GRV~~Q~~V~~Q~~KNS~~ISACHGR~~DVSR~~LPNGAYIL~~KVEGYTTK~~INIVH~~

Fig. 1b

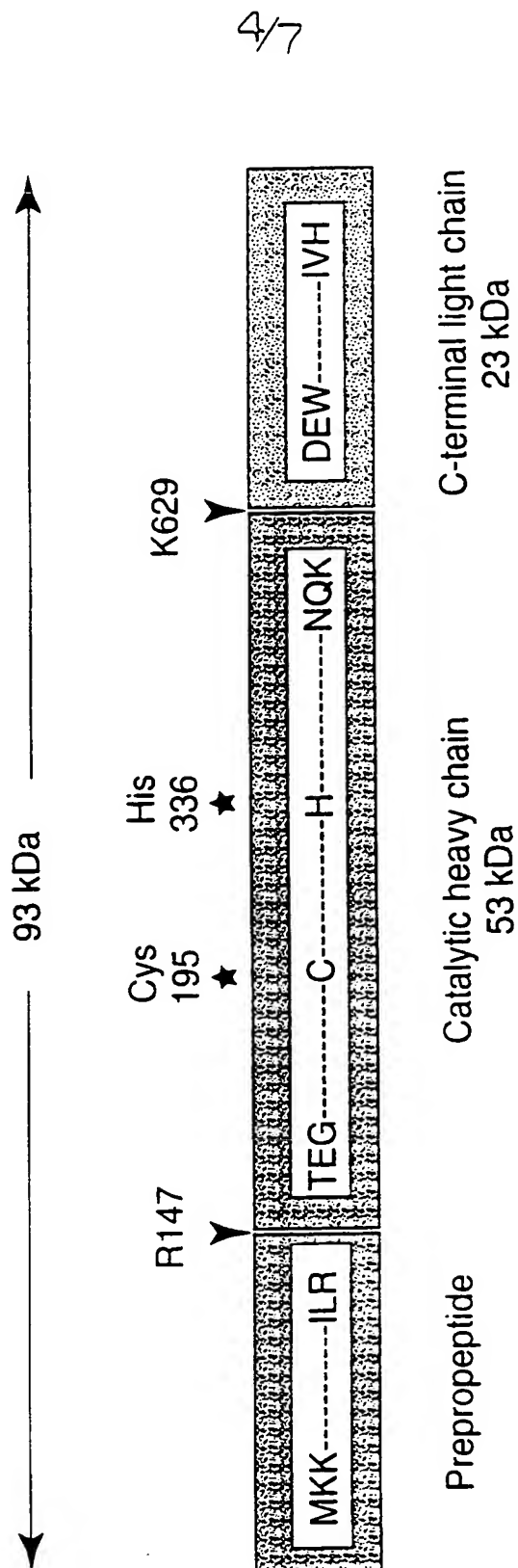


Fig. 2

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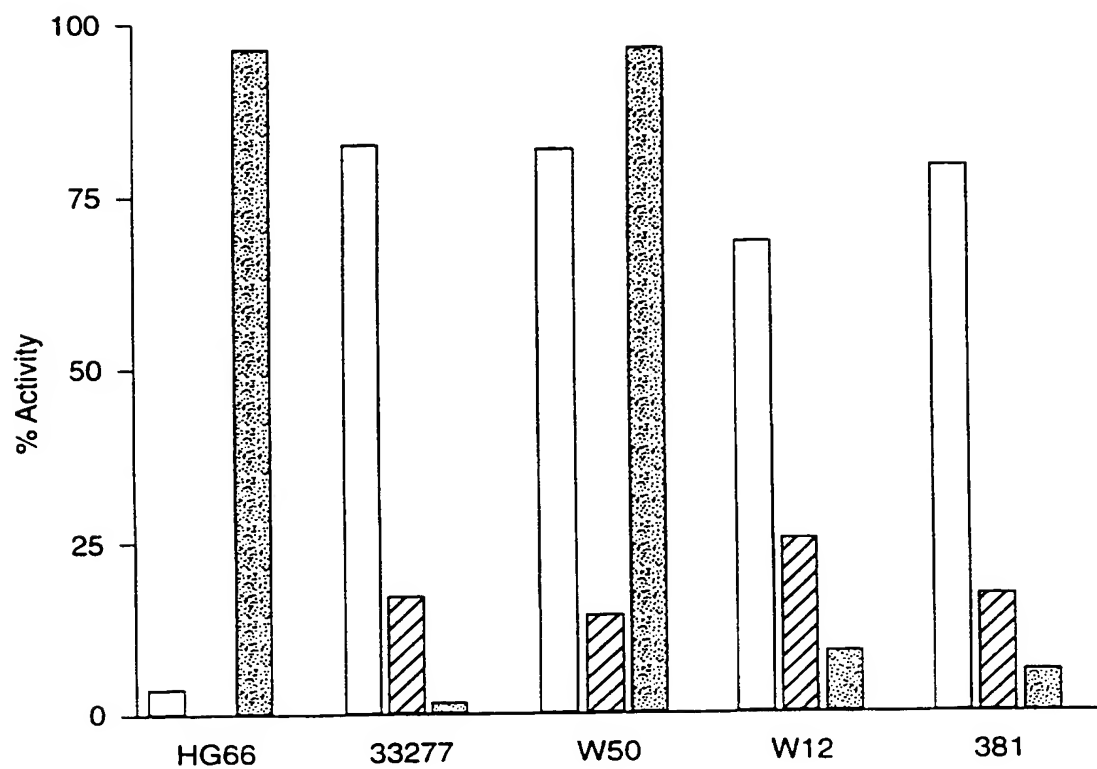


Fig. 3

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MPSSVSWGILLAGLCCLVPVSLAEDPQGDAAQKTDTSHHQDHP
TFNKITPNLAFAFSLYRQLAHQSNSTNIFFSPVSIATAFAMLSLGTKADTHDEILEG
LNFNLTEIPEAQIHEGFQELLRTLNPDSQLQLTTGNGLFLSEGLKLVDFLEDVKKL
YHSEAF TVNFGDTEEAKKQINDYVEKGTQGKIVDLVKELDRDTV FALVNYIFFKGKWE
RPFVVDTEEEEDFHVDQVTTVKVPMMKRLGMFNIQHCKKLSSWVLLMKYLG NATAIFF
LPDEGLQHLLENELTHDIITKFLENEDRRSASLHLPKLSITGTYDLKSVLGQLGITKV
FSNGADLSGVTEEAPLKLSKAVHKAVLTIDEKGTEAAGAMFLEAIPMSIPPEVKFNKP
FVFLMIEQNTKSPLFMGKVVNPTQK

SEQ ID NO:3

Fig. 4

GAMFLEAIPMSIPPE

SEQ ID NO:4

Fig. 6

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Fig. 5

ATGAAAAAAGTTTTCTTTTAGCCATAGTAATGCTCTTTGGCATTGCCATGCAGGGACAT
TCTGCTCCGGTTACGAAAGAGCGAGCTTTGAGTCTGGCTCGGCTGGCTTTGCGACAGGTA
TCCTTGCGAATGGGACAAACAGCAGTATCTGACAAGATTTCCATCGATTACGTTTATCGG
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AGCCGACTTCCTAATGGGGCCTACATCCTTAAGGTGGATGGATATACGACGAAAATAAAT
ATAGTGCCTAA

SEQ ID NO:2

INTERNATIONAL SEARCH REPORT

International Classification No.

PCT/US 00/10574

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/57 C12N9/64 C12Q1/37 A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	POTEMPA J ET AL: "THE INACTIVATION OF HUMAN PLASMA ALPHA-1 PROTEINASE INHIBITOR BY PROTEINASES FROM STAPHYLOCOCCUS-AUREUS" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 261, no. 30, 1986, pages 14330-14334, XP002154472 ISSN: 0021-9258 the whole document --- -/--	1, 2, 4-7, 29

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Van der Schaal, C

INTERNATIONAL SEARCH REPORT

International Publication No.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
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information on patent family members

Inter. Publication No
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